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Neuroinflammation and alpha-synuclein oligomer toxicity in the pathogenesis of Lewy body dementia and Parkinson's disease

Thesis submitted by

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ABSTRACT

Aggregated α -synuclein has emerged as the core constituent of the typical neuronal inclusions found in Parkinson's disease and Lewy body dementia. Therefore, huge efforts have been made to unveil the mechanisms underlying α -syn toxicity. Accumulating evidence suggests extracellular α -synuclein oligomers (α -synOs) as potential culprits involved in the neurodegenerative process. To elucidate the pathways mediating α -synO non-cell-autonomous actions, several mechanisms including uncontrolled neuroinflammatory responses and protein-protein interactions have been put forward.

Through an acute model based on the intracerebroventricular (ICV) injection of α -synuclein monomers, oligomers or fibrils in C57BL/6N mice we demonstrate that only α -synOs impair memory establishment in association with glial activation. Furthermore, our findings identify neuroinflammation as a driving force of α -synO detrimental action on memory, and the involvement of the Toll-like receptor 2. Based on recent data depicting the cellular prion protein (PrP^C) as an α -synO interactor, we have further investigated its role in fostering α -synO harmful activities. We found that PrP^C does not mediate α -synO toxicity *in vitro* or α -synO-induced memory deficiency *in vivo*. In fact, PrP^C knock-out mice ICV injected with α -synOs display both memory impairment and gliosis. Consistently, our *in vitro* biochemical studies do not reveal any direct PrP^C- α -synO binding.

To evaluate the influence of neuroinflammation on PD pathogenesis, we have developed a “double-hit” approach. Using an acute mouse model based on the peripheral administration of lipopolysaccharide (LPS) and subsequent ICV injection of α -synOs at an inactive dose, we demonstrate that the LPS induces a long-lasting neuroinflammatory response enhancing α -synO actions. Moreover, we show that the LPS peripheral administration worsens cognitive deficits even in an A53T PD mouse model. Altogether, by identifying neuroinflammation as an α -synO-mediator and as a factor influencing the

initiation/progressions of PD, we highlight it as a valuable research topic to identify potential targets for developing new therapeutic strategies.

PREFACE

The work described herein was performed at the Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, from October 2015 to September 2019. The PhD research project was conducted under the supervision and direction of Dr. Gianluigi Forloni (director of the studies) and Prof. Andrey Y. Abramov (external supervisor).

DECLARATION

This PhD research project has not been submitted in whole or in part for a degree or diploma or other qualification to any other university.

The experimental work described here was performed by me, Pietro La Vitola, and includes work carried out in collaboration with:

- Prof. Loredano Pollegioni and Dr. Laura Caldinelli, Department of Biotechnology and Life Sciences, Università degli Studi dell'Insubria, Varese, Italy, who provided and characterised monomeric alpha-synuclein used in our experiments.

- Dr. Laura Colombo, Department of Molecular Biochemistry and Pharmacology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, which performed Atomic Force Microscopy analyses of our alpha-synuclein aggregates.

- Dr Milica Cerovic, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, who performed extracellular field recording experiments.

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- Dr. Marco Gobbi and Dr. Marten Beeg, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, who performed Surface Plasmon Resonance experiments to investigate the cellular Prion protein-alpha-synuclein oligomer binding.

- Dr. Roberto Chiesa and Dr. Elena Restelli, Department of Neuroscience, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, which performed primary hippocampal cell cultures and the analyses of their viability after exposure to alpha-synuclein oligomers.

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My most important thanks go to my parents, you always have been supportive and believing in me.

**CANDIDATE PUBLICATIONS PRECEDING THE WORK
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“The Continuing Failure of Bexarotene in Alzheimer's Disease Mice”

Balducci C, Paladini A, Micotti E, Tolomeo D, **La Vitola P**, Grigoli E, Richardson JC, Forloni G.

J Alzheimers Dis. 2015; 46(2):471-82. doi: 10.3233/JAD-150029.

“Multifunctional liposomes reduce brain β -amyloid burden and ameliorate memory impairment in Alzheimer's disease mouse models”

Balducci C, Mancini S, Minniti S, **La Vitola P**, Zotti M, Sancini G, Mauri M, Cagnotto A, Colombo L, Fiordaliso F, Grigoli E, Salmona M, Snellman A, Haaparanta-Solin M, Forloni G, Masserini M, Re F.

J Neurosci. 2014 Oct 15; 34(42):14022-31. doi: 10.1523/JNEUROSCI.0284-14.2014.

“An N-terminal fragment of the prion protein binds to amyloid- β oligomers and inhibits their neurotoxicity in vivo”

Fluharty BR, Biasini E, Stravalaci M, Scip A, Diomedea L, Balducci C, **La Vitola P**, Messa M, Colombo L, Forloni G, Borsello T, Gobbi M, Harris DA.

J Biol Chem. 2013 Mar 15; 288(11):7857-66. doi: 10.1074/jbc.M112.423954. Epub 2013 Jan 28.

**CANDIDATE PUBLICATIONS EMANATING FROM THE WORK
DESCRIBED IN THIS THESIS**

“Cellular prion protein neither binds to alpha-synuclein oligomers nor mediates their detrimental effects”

La Vitola P, Beeg M, Balducci C, Santamaria G, Restelli E, Colombo L, Caldinelli L, Pollegioni L, Gobbi M, Chiesa R, Forloni G.

Brain. 2019 Feb 1; 142(2):249-254. doi: 10.1093/brain/awy318.

“Alpha-synuclein oligomers impair memory through glial cell activation and via Toll-like receptor 2”

La Vitola P, Balducci C, Cerovic M, Santamaria G, Brandi E, Grandi F, Caldinelli L, Colombo L, Morgese MG, Trabace L, Pollegioni L, Albani D, Forloni G.

Brain Behav Immun. 2018 Mar; 69:591-602. doi: 10.1016/j.bbi.2018.02.012. Epub 2018 Feb 16.

“Oligomeropathies and pathogenesis of Alzheimer and Parkinson's diseases”

Forloni G, Artuso V, **La Vitola P**, Balducci C.

Mov Disord. 2016 Jun; 31(6):771-81. doi: 10.1002/mds.26624. Epub 2016 Mar 31

**CANDIDATE PUBLICATIONS EMANATING FROM WORK NOT
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“Doxycycline counteracts neuroinflammation restoring memory in Alzheimer's disease mouse models”

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Neurobiol Aging. 2018 Oct; 70:128-139. doi: 10.1016/j.neurobiolaging.2018.06.002. Epub 2018 Jun 12.

“Toll-like receptor 4-dependent glial cell activation mediates the impairment in memory establishment induced by β -amyloid oligomers in an acute mouse model of Alzheimer's disease”

Balducci C*, Frasca A*, Zotti M*, **La Vitola P**, Mhillaj E, Grigoli E, Iacobellis M, Grandi F, Messa M, Colombo L, Molteni M, Trabace L, Rossetti C, Salmona M, Forloni G.

Brain Behav Immun. 2017 Feb; 60:188-197. doi: 10.1016/j.bbi.2016.10.012. Epub 2016 Oct 14.

LIST OF ABBREVIATIONS

α -syn	Alpha-synuclein
α -synOs	Alpha-synuclein oligomers
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
AFM	Atomic force microscopy
AMPArs	Amino-3-hydroxy-5-methyl-1-4-isoxazolepropioni receptors
ANOVA	Analyses of variance
ATP	Adenosine three phosphate
A β Os	Amyloid- β oligomers
Ca ²⁺	Calcium
CaM	Calmodulin
CaMKII	Adenylate cyclase and Ca ²⁺ /CaM-protein kinase II
cAMP	Cyclic adenosine monophosphate
CaN	Calcineurin
CNS	Central nervous system
COX1	Cyclooxygenase I
COX2	Cyclooxygenase II
CREB	Response element binding protein
CSF	Cerebrospinal fluid
DAMPs	Damage associated molecular patterns
DI	Discrimination index
DLB	Dementia with Lewy Bodies
EIF4G1	Eukaryotic translation initiation factor 4-gamma 1
ER	Endoplasmic reticulum

GPe	<i>Globus Pallidus pars externa</i>
GPi	<i>Globus Pallidus pars interna</i>
IBF	Ibuprofen
ICV	Intracerebroventricular
IFN γ	Interferon gamma
IL-1 β	Interleukin 1 β
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-13	Interleukin 13
Indo	Indomethacin
iNOS	Inducible nitric oxide synthase
IP	Intraperitoneal
iPSC	Induced pluripotent stem cells
K ⁺	Potassium
LB	Lewy body
LBD	Lewy bodies dementia
LBP	Lipid binding protein
LDH	Lactate dehydrogenase
LN	Lewy neurite
LPS	Lipopolysaccharide
LRRK2	Leucine reach repeat kinase 2
LTP	Long term potentiation
Mg ²⁺	Magnesium
MPTP	1-methyl,-4-phenyl-1,2,3,6-tetrahydropyridine
MTT	Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MWM	Morris-water maze
Na ⁺	Sodium
NAC	Non-amyloidogenic component of amyloid plaques
NACP	Precursor of non-amyloidogenic component of amyloid plaques
NMDARs	N-methyl-D-aspartate receptors
NO	Nitric oxide
NORT	Novel object recognition task
NSAIDs	Non-steroidal anti-inflammatory drugs
NTG	Non-transgenic
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer saline
PD	Parkinson's disease
PDD	Dementia associated to Parkinson's disease
PINK1	Phosphatase and tensin homolog-induced kinase 1
<i>Prnp^{0/0}</i>	Cellular prion protein knock-out
PrP ^C	Cellular prion protein
PRRs	Pattern recognition receptors
RBD	Rapid eye movement sleep behaviour disorder
ROS	Reactive oxygen species
RT	Room temperature
SN	<i>Substantia nigra</i>
SNpc	<i>Substantia Nigra pars compacta</i>
SNpr	<i>Substantia Nigra pars reticulata</i>
SPR	Surface Plasmon Resonance
STN	<i>Nucleus subthalamicus</i>
TBS	Tris-HCl-buffered saline

Tg	Transgenic
TGF β	Transforming growth factor β
Th	Tyrosine hydroxylase
TIF	Total insoluble fraction
TIR	Toll-interleukin 1 receptor
TLR	Toll-like receptor
TLR4 ^{0/0}	Toll-like receptor 4 knock-out
TNF α	Tumour Necrosis Factor α
Veh	Vehicle
VPS35	Vesicular protein sorting 35
WT	Wild-type
YFP	yellow fluorescent protein

Table of Contents

ABSTRACT	I
PREFACE	III
DECLARATION	IV
ACKNOWLEDGEMENTS	V
CANDIDATE PUBLICATIONS PRECEDING THE WORK DESCRIBED IN THIS THESIS	VI
CANDIDATE PUBLICATIONS EMANATING FROM THE WORK DESCRIBED IN THIS THESIS	VII
CANDIDATE PUBLICATIONS EMANATING FROM WORK NOT PERTAINING WITH THIS THESIS	VIII
LIST OF ABBREVIATIONS	IX
TABLE OF CONTENTS	XIII
LIST OF FIGURES.....	XIX
LIST OF TABLES	XX
INTRODUCTION	1
CHAPTER I	1
1.1 A brief overview on the long history of Parkinson's disease	2
1.2 Neuropathological features	3
1.2.1 The nigrostriatal pathway	6
1.3 Clinical features	8
1.3.1 Non-motor symptoms of Parkinson's disease	9
1.3.1.1 Cognitive impairment in Parkinson's disease	10
1.3.2 Dementia with Lewy bodies	11
1.3.3 Dementia in Parkinson's disease and Dementia with Lewy bodies	13
1.4 The Braak staging of Parkinson's disease	15
1.4.1 The controversial role of Lewy Bodies	16
1.5 Epidemiology of Parkinson's disease	17
1.5.1 Epidemiology of Lewy body dementia	19
1.6 Risk factors for Parkinson's disease.....	19
1.6.1 Modifiable risk factors	19

1.6.2 Non-modifiable risk factors	21
1.6.2.1 Genes involved in autosomal dominant forms of PD	22
1.6.2.2 Genes involved in autosomal recessive forms of PD	25
1.7 Diagnosis of Parkinson's disease	27
1.8 Diagnosis of Dementia with Lewy bodies and Dementia associated to Parkinson's disease	29
CHAPTER II	32
2.1 The synuclein family: a brief overview	33
2.2 Alpha-synuclein: a key protein in PD and α -synucleinopathies	34
2.3 Insight into α -syn structure and physiological functions	35
2.3.1 Memory, synaptic plasticity and LTP	41
2.4 The aggregation of α -syn and the oligomer hypothesis	43
2.5 Intracellular mechanisms involved in α -synO toxicity	47
2.6 Evidence for α -synOs in extracellular fluids and its implications	50
2.6.1 The non-cell-autonomous action of α -synOs	52
2.7 Alpha-synuclein oligomers and cognitive impairment	54
CHAPTER III	57
3.1 Evidence for the role of inflammation in α -synucleinopathies	58
3.2 Astrocytes and microglial cells are key players in the healthy brain	62
3.3 General characteristic of microglial cells	65
3.3.1 The dual role of microglial activation	66
3.4 Brain and peripheral inflammation: a strong connection	60
3.5 The Toll-like receptors	71
3.6 Altered expression of TLR2 and 4 in Parkinson's disease	73
3.6.1 Experimental evidence for TLR2 and TLR4 involvement in α -syn detrimental effects	74
AIMS OF THE STUDY	77
CHAPTER IV	77
MATERIALS AND METHODS	80
CHAPTER V	80
5.1 Mice	81
5.2 ICV cannulation	81
5.3 Alpha-syn assembly preparations	82
5.4 Atomic force microscopy	82

5.5 Western blotting of α -synOs	83
5.6 Alpha-synOs and vehicle ICV injection	83
5.7 Drugs and treatment	84
5.8 Novel object recognition task (NORT) and open field	85
5.9 Y-maze test	85
5.10 Morris-water maze (MWM) test	86
5.11 Beam-walk test	86
5.12 Open field	86
5.13 Extracellular field recordings	87
5.14 Immunohistochemistry and immunofluorescence	88
5.15 Nissl staining	90
5.16 Western blotting of synaptic proteins	90
5.17 Hippocampal neuron cultures and determination of α -synO toxicity	91
5.18 Surface Plasmon Resonance.....	92
5.19 Statistical analysis	93
RESULTS	94
CHAPTER VI	94
Alpha-synOs acutely impair memory whereas monomers and fibrils were ineffective.....	94
6.1 Aim of the study and <i>in vivo</i> experimental design	95
6.2 Results	96
6.2.1 Alpha-synOs specifically induce memory deficiency in C57BL/6 naïve mice and impair hippocampal LTP on brain slices	96
6.2.2 ICV injected α -syn spreads across the hippocampus	102
6.2.3 Alpha-synO-mediated recognition memory impairment is transient and not associated with hippocampal alterations at both neuronal and synaptic level.....	104
6.3 Discussion	107
CHAPTER VII.....	109
Alpha-synO-mediated memory impairment is dependent on glial activation and TLR2.....	109
7.1 Aim of the study and experimental design	110
7.2 Results	113
7.2.1 Alpha-synO-mediated memory impairment is associated with hippocampal neuroinflammation.....	113

7.2.2 Neuroinflammation is a crucial mechanism involved in α -synO-mediated memory damage	115
7.2.3 Alpha-synOs lead to gliosis in the hippocampus whereas monomers and fibrils are ineffective	120
7.2.4 Alpha-synO-mediated memory impairment is TLR2-dependent.....	121
7.3 Discussion.....	123
CHAPTER VIII	128
Alpha-synO harmful actions are not dependent on the cellular Prion protein	128
8.1 Aim of the study and experimental approaches	129
8.2 Results	131
8.2.1 Alpha-synO-mediated detrimental effects are PrP ^C -independent	131
8.2.2 Alpha-synOs and PrP ^C do not directly interact	136
8.3 Discussion.....	137
CHAPTER IX	140
Peripherally induced neuroinflammation influences α -synO harmful actions and the PD phenotype in an acute and transgenic mouse model	140
9.1 Aim of the study and experimental design.....	141
9.2 Results	144
9.2.1 LPS-preconditioning enhances α -synO-induced toxicity in primary hippocampal neuronal cultures	144
9.2.2 Establishment of the two challenges for the “double-hit” experimental model	146
9.2.3 Peripherally LPS-induced neuroinflammation enhances α -synO-mediated memory damage	149
9.2.4 Microglial cells and astrocytes differentially respond in the “double-hit” acute mouse model	151
9.2.5 Peripheral administration of LPS negatively influences cognitive performances in the A53T PD-related transgenic mouse model	156
9.2.6 Peripheral LPS induces a different response of microglial and astroglial cells in the A53T PD-related transgenic mouse model	159
9.2.7 Peripheral LPS does not affect motor behaviour and dopaminergic neurodegeneration in A53T mice	162
9.3 Discussion.....	165

SUMMARY AND FINAL REMARKS	168
CHAPTER X	168
10.1 Summary and final remarks	169
10.1.a Conclusions	178
BIBLIOGRAPHY	181

LIST OF FIGURES

Figure 1	2
Figure 2	4
Figure 3	5
Figure 4	5
Figure 5	7
Figure 6	15
Figure 7	18
Figure 8	23
Figure 9	33
Figure 10	35
Figure 1 1	36
Figure 1 2	39
Figure 1 3	44
Figure 1 4	47
Figure 1 5	53
Figure 1 6	63
Figure 1 7	68
Figure 1 8	79
Figure 1 9	96
Figure 20	97
Figure 21	101
Figure 22	103
Figure 23	104
Figure 24	105
Figure 25	106
Figure 26	112
Figure 27	113
Figure 28	114
Figure 29	116
Figure 30	117
Figure 31	118
Figure 32	119

Figure 33	121
Figure 34	122
Figure 35	123
Figure 36	130
Figure 37	132
Figure 38	133
Figure 39	134
Figure 40	135
Figure 41	136
Figure 42	143
Figure 43	144
Figure 44	145
Figure 45	148
Figure 46	150
Figure 47	151
Figure 48	152
Figure 49	153
Figure 50	154
Figure 51	155
Figure 52	158
Figure 53	161
Figure 54	164

LIST OF TABLES

Table 1	14
Table 2	28
Table 3	30
Table 4	60

Introduction

Chapter I

1.1 A brief overview on the long history of Parkinson's disease

The first partial clinical descriptions of Parkinson's disease (PD) can be found in ancient Indian and Chinese populations, which have described both PD motor symptoms and therapeutic recommendations since 5000 B.C (Zhang *et al.*, 2006a; Goetz, 2011; Goedert *et al.*, 2017).

Nevertheless, the complete clinical report of PD cardinal motor symptoms was provided in 1817 by the London physician James Parkinson. In his monograph “Essay on the Shaking Palsy” he punctually described a series of six patients characterised by movement disorders which came to bear his name: *“involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forward, and to pass from a walking to a running pace: the senses and intellect being uninjured”* (Goedert *et al.*, 2017; Fahn, 2018)

A thorough description of PD was provided 50 years later by Jean-Martin Charcot (1872). He identified bradykinesia as a cardinal feature of the pathology and distinguished two prototypes of the disease: the tremulous and the rigid/akinetic type. Moreover, Charcot rejected the first designation of the disease (paralysis agitans or shaking palsy), and suggested the term already being used (PD). In fact, he observed that PD patients were not weak and did not necessarily display tremor (Charcot, 1875). The most important Charcot's contribution to the study of PD is the definition of the different types of the disorder. For instance, he identified an atypical form of PD without tremor and with a stiff and extended posture (Figure 1).



Figure 1. Charcot's drawing of typical and atypical PD. Typical PD showing a flexed posture (left) and atypical (right) PD with absence of tremor and extended posture (modified from Goetz, 2011).

At the beginning of the XX century, Brissaud identified the *Substantia nigra* (SN) as the crucial brain region affected in PD. Soon after, Friedrich Lewy reported the typical intracellular inclusions in several brain regions (1912), that were named ‘Lewy bodies (LB)’ by Tretiakoff in 1919 (Goedert *et al.*, 2017)

Forty years later the studies by Carlsson and co-workers indicated the deficiency of the neurotransmitter dopamine as responsible of the motor symptoms in PD (Carlsson *et al.*, 1958; Carlsson *et al.*, 2001). This possibility was subsequently confirmed by the evidence of dopamine being reduced in PD patients (Ehringer and Hornykiewicz, 1960), and by observations that SN, a brain region enriched in dopaminergic neurons (Dahlstrom and Fuxe, 1964), was particularly damaged in PD patients.

From the description of Parkinson and Charcot, motor symptoms clearly emerge as cardinal PD features. Although the relevance of motor symptoms in PD is widely accepted, nowadays non-motor symptoms acquire a significant relevance in terms of factors that largely impact life quality. Among non-motor symptoms, cognitive deficits have gained more and more attention as they have become a well-accepted parameter for differentiating between dementia associated to PD (PDD) and Dementia with Lewy Bodies (DLB) (McKeith *et al.*, 2004; Galasko, 2017). PD, PDD and DLB all share common neuropathological features including LBs, which at present has a history longer than one century.

1.2 Neuropathological features

Loss of dopaminergic neurons in *Substantia Nigra pars compacta* (SNpc) and intraneuronal proteinaceous inclusions are defining hallmarks of the progressive neurodegenerative disorder PD.

The depletion of dopaminergic neurons in SNpc (Figure 2) accounts for changes in the nigrostriatal pathway which result in the typical PD motor symptoms (Lang and Lozano, 1998; Sveinbjornsdottir, 2016). Dopaminergic neurons in SNpc are particularly affected in

PD, however other specific neurons (glutamatergic, GABA-ergic, cholinergic, noradrenergic and adrenergic) in selective brain regions can be progressively damaged. For instance, extranigral abnormalities have been observed within the entorhinal cortex, in the CA2 region of the hippocampus, in the amygdala, in the hypothalamus, in the *nucleus basalis* of Meynert and *locus caeruleus* (Lang and Lozano, 1998; Braak and Braak, 2000).

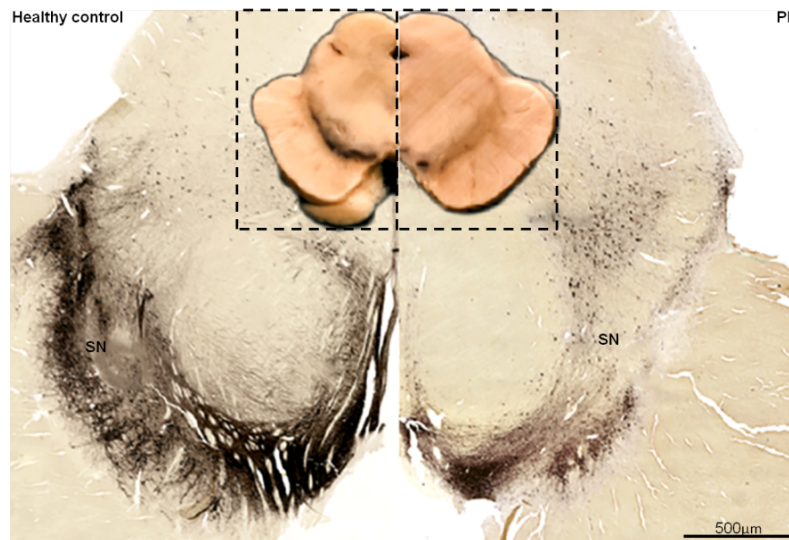


Figure 2. Depletion of dopaminergic neurons in SN from a PD patient compared to healthy control. Macroscopic (inset) and coronal section of the midbrain upon immuno-staining for the limiting enzyme Tyrosine hydroxylase (Th) involved in dopamine synthesis. PD patients (right) are characterised by a significant loss of Th⁺ neurons compared to healthy controls (left). Modified from Poewe *et al.*, 2017.

The intracellular inclusions typically observed in post-mortem PD brains can be classified into Lewy bodies (LBs) or Lewy neurites (LNs) based on their localisation in the somata or neuronal processes respectively. Two distinct LBs can be identified. The brainstem-type (classical) LBs are easily seen upon haematoxylin and eosin staining. They appear as single or multiple spherical inclusions with a diameter between 5 to 25 µm and a central dense eosinophilic core surrounded by a peripheral pale halo (Figure 3A). Cortical-type LBs (Figure 3B) are eosinophilic like brainstem type LBs, but they have an irregular shape and they lack a dense central core and halo (Lang and Lozano, 1998; Wakabayashi *et al.*, 2013).

Aside of classical and cortical LBs, a third intracellular inclusion type can be observable in the pigmented neurons of SNpc, the “pale bodies” (Figure 3C). Pale bodies are less eosinophilic with a glassy area and without halo. Pale bodies frequently occur with LBs in the same neurons (Figure 3C) and it has been proposed that pale bodies are linked to LBs formation (Wakabayashi *et al.*, 2013). LBs have been detected for a long time by anti-ubiquitin antibodies. In fact, both brainstem type and cortical LBs can be strongly immunostained with ubiquitin (Kuzuhara *et al.*, 1988),

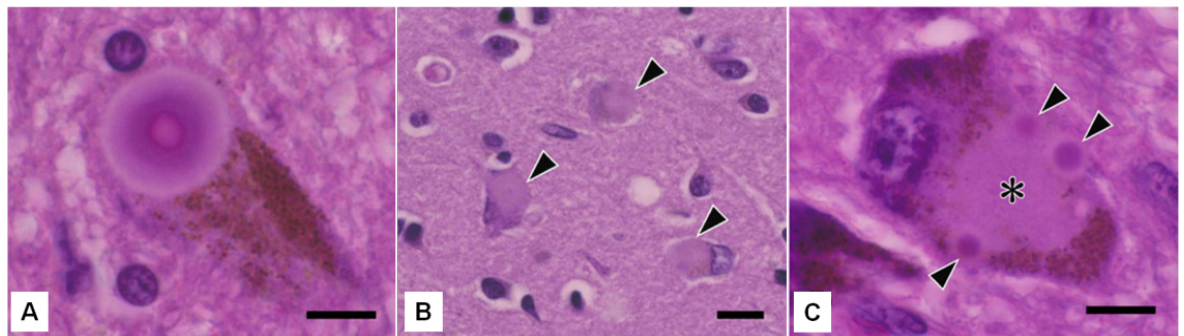


Figure 3. Haematoxylin and eosin staining of LBs. (A) Classical LBs in the SN. **(B)** Cortical LBs indicated by arrowheads in the temporal cortex. **(C)** Pale body (asterisk) and classical LBs (arrowheads) within SN neurons. Scale bar 10µm. Modified from Wakabayashi *et al.*, 2013.

More than 90 elements have been identified in both LBs and LNs (Wakabayashi *et al.*, 2013), among which alpha-synuclein (α -syn) emerges as a central component and building block (Figure 4) (Spillantini *et al.*, 1997; Spillantini *et al.*, 1998; Baba *et al.*, 1998).

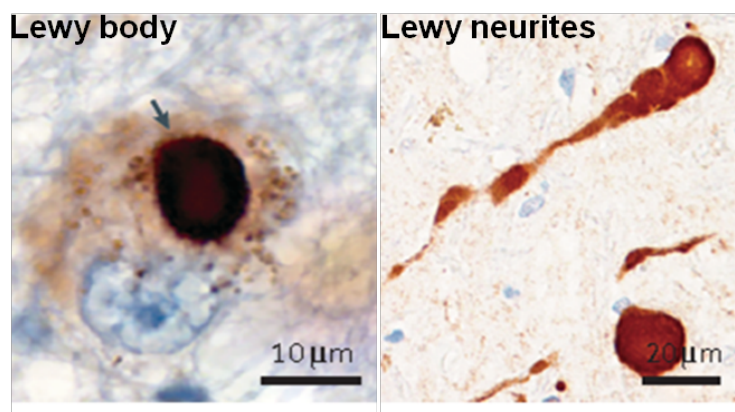


Figure 4. α -syn is the main constituent of LBs and LNs. α -syn immunostaining of a post-mortem PD brain reveals the presence of α -syn in both LBs and LNs (Modified from Poewe *et al.*, 2017).

1.2.1 The nigrostriatal pathway

The idea of lesions in the SN as anatomical substrate of PD is due to Edouard Brissaud in 1894, who knew well the previous study of Charcot as well as the work of Blocq and Marinesco (Spillantini and Goedert, 2018). The microscopic and macroscopic SNpc alterations reported by Trétiakoff, the reduction in dopamine levels in the striatum and in the SNpc (Przedboski, 2017), along with the efficacy of L-DOPA in alleviating motor deficits in PD patients, points towards an involvement of the nigrostriatal pathway in PD (Fahn, 2015). This hypothesis had been built over the 20th century, when researchers established the link between dysfunctions in the basal ganglia-thalamo-cortical circuit and the motor defects typical of PD. In these studies, discrete lesions of the basal ganglia and chemical destruction of the *nucleus subthalamicus* (STN) led to abrogation of parkinsonisms and allowed the dissection of the functional neuroanatomy of the basal ganglia (Meyers, 1942; Bergman *et al.*, 1990). Basal ganglia refers to those grey matter structures at the base of the cerebral hemisphere, encompassing a network of connected subcortical nuclei such as the *striatum* (*nucleus caudatus* and *putamen*), the SNpc and the *Substantia Nigra pars reticulata* (SNpr), the STN, and the *Globus Pallidus pars externa* (GPe) and *pars interna* (GPi). The motor circuit of the basal ganglia is involved in the regulation of movements through the synergy of the nigrostriatal, and the “direct” and “indirect” motor loop pathways (Figure 5 A). The motor cortex projects excitatory input to the *striatum* reflecting cortical somatotopic organisation (Takada, 1998). The *striatum* in turn, is modulated through dopamine release by the SNpc. The nigrostriatal pathway acts on two types of dopamine receptors, that define the overall final output from the basal ganglia to the motor nuclei. While the excitatory D1 receptors are involved in the “direct” pathway which facilitates voluntary movements, the inhibitory effect of D2 receptors in the “indirect” pathway prevents unwanted movements. In the “direct” pathway, the excitatory effect of dopamine on D1 (Figure 5A) triggers the release of GABA that inhibits the GABAergic neurons of the GPi/SNpr (Figure 5A),

decreasing the final inhibition on the motor nuclei. This way the direct pathway generates a net excitatory effect that promotes movement initiation. On the other hand, in the “indirect” pathway dopamine dampens striatal GABAergic activity through D2 receptors. Because the “direct” pathway only involves a single synapse is commonly referred to as monosynaptic, whereas the “indirect” one includes polysynaptic projections that cross the GPe and the STN before reaching the output nuclei (GPi/SNpr) (Figure 5 A). The GPe, via GABAergic stimuli, acts as a negative regulator of the “direct” pathway by modulating the glutamatergic activity of the STN (red arrows), that ultimately reaches the output nuclei. Activated GPi/SNpr leads then to a net inhibition of movement (Galvan *et al.*, 2015).

In PD patients, the dopaminergic nigrostriatal pathway progressively degenerates (Figure 5B), resulting in the decrease of dopamine levels. As a consequence, the activation of the “direct” and the inhibition of the “indirect” pathways are drastically reduced, leading to an overall strong inhibition of the motor activity (Przedbosrki, 2017).

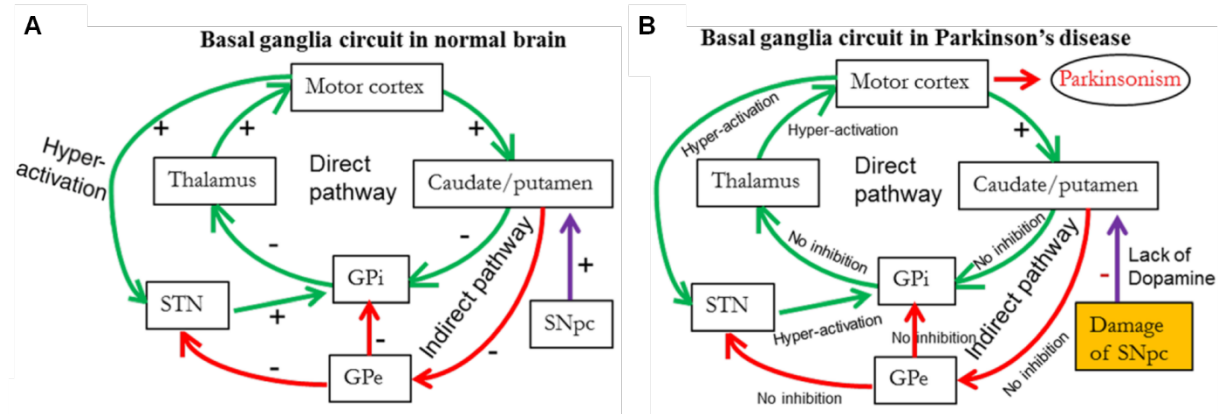


Figure 5. Schematic representation of the “direct” and “indirect” pathways of the basal ganglia motor circuits on healthy control (A) and PD patients (B). Green arrows represent excitatory pathways whereas red arrows are inhibitory. Modified from Maii *et al.*, 2017.

1.3 Clinical features

PD is a debilitating disease with a progressive worsening of symptoms that primarily affect voluntary movements. Although motor symptoms are cardinal features of PD, a variety of non-motor symptoms have been described. Non-motor symptoms contribute to decrease the life quality and they can appear before the onset of motor-symptoms or during the progression of the disease (Sveinbjornsdottir, 2016). The dopaminergic neuronal loss in SNpc has been estimated to be 60 to 80% at the onset of motor symptoms (Fearnley *et al.*, 1991; Chung *et al.*, 2001). At first, those symptoms appear asymmetrically, affecting the other side of the body within a few years. Postural changes, tremor, muscular rigidity, bradykinesia, and gait instability are defined as cardinal PD motor features. Postural deformities including flexion of the thoracic or lumbar spine, and forward flexion of the head and neck cause the body posture to become stooped (Sveinbjornsdottir, 2016). Resting tremor is one of the most obvious features of the disease, being one of the first and more distressing symptoms reported by patients. In most cases, a resting pill-rolling type tremor of the hands is present, whereas legs are involved only occasionally. Furthermore, about 60% of patients experience tremor during voluntary movements (Heusinkveld *et al.*, 2018). Bradykinesia describes slowness in carrying out rather than initiating movements, and it accounts for expressionless face (*hypomimia*) and smaller handwriting (*micrographia*). Conversely, gait disturbances include shuffling, blocking, freezing of movements, and festination, where steps become progressively smaller and rapid leading to loss of balance and falls (Virmani *et al.*, 2015). Less common in PD is dystonia, a non-voluntary contraction of antagonist muscles leading to spasms with different degree of pain. Contractions can be associated with abnormal movements and posture. Dystonia is more often a consequence of PD treatment, although it rarely occurs in early onset PD patients (Tolosa and Compta, 2006).

1.3.1 Non-motor symptoms of Parkinson's disease

As mentioned above, a variety of non-motor symptoms may appear in PD patients before the onset of motor symptoms and before the diagnosis. Particularly, it has been reported that non-motor symptoms may affect patients up to 10 years before the diagnosis of PD. These data allow the determination of a target temporal window, defined as pre-motor or prodromal phase, for preventive and disease-modifying treatments aimed at slowing or blocking the pathology progression (Schrag *et al.*, 2015; Kalia and Lang, 2015).

Like motor symptoms, non-motor ones get worse over the course of the pathology and they are described by patients as more troublesome than classical PD motor features (Sveinbjornsdottir, 2016).

A large amount of various non-motor clinical features has been reported in PD patients including sensory dysfunctions, neuropsychiatric features, sleep disorders and autonomic dysfunctions (Kalia and Lang, 2015; Schapira *et al.*, 2017). Sensory symptoms are virtually experienced by all PD patients as a part of their prodromal phase (Schapira *et al.*, 2017). Among these, olfactory deficit has a higher prevalence being observed in 90% of subjects and it is generally considered as useful early pre-motor PD marker (Poewe, 2008; Schapira *et al.*, 2017). Visual disturbances, and particularly visual hallucination, initially considered as an adverse effects of drug treatment in advanced PD, are now depicted as typical prodromal PD features and are reported in untreated patients. Of note, visual hallucination can be included among neuropsychiatric features of PD and is considered as a predictor of cognitive decline in the later stage (Jankovic, 2008; Schapira *et al.*, 2017).

Neuropsychiatric manifestations (*e.g.* anxiety, depression. sleep disorders...) occur both in the pre-motor and in the late phase of PD (Poewe, 2008). Anxiety is experienced by 60% of patients and comprises generalised anxiety, panic attacks and social phobias. Although anxiety is generally accompanied by depression, the latter may also occur alone. PD-related

depression is milder than depression occurring in non-PD affected subjects and it frequently involves apathy and anhedonia (Schapira *et al.*, 2017).

An increasing interest in non-motor PD features has been recently growing for sleep disorders. Sleep and wakefulness disturbances affect most PD patients and their prevalence increase with the disease duration and progression. Of note, rapid eye movement sleep behaviour disorder (RBD) is a parasomnia characterised by aberrant and abnormal behaviours (talking, sitting up to bed, gesturing...) which take place during rapid eye movement sleep. The interest in RBD comes from the evidence that it can appear even 12-14 years earlier than motor manifestations. In addition, RBD is predictive of cognitive deficits. In fact, patients with RBD show an increased risk of dementia (Kalia and Lang; 2015; Schapira *et al.*, 2017).

Beside the various non-motor disturbances here reported, compelling data indicate that PD patients also experience dysfunctions affecting the autonomic system, which precede the onset of motor symptoms but become more frequent as the disease progresses (Poewe, 2005; Schapira *et al.*, 2017). Autonomic dysfunctions include bladder disturbances, gastrointestinal dysfunctions (excessive salivation, dysphagia, impaired gastric emptying, constipation and bacterial overgrowth).

Of note, non-motor PD-related symptoms are now recognised as component of PD that is not solely a CNS disorder but, more likely, a multisystem pathology (Lee and Koh, 2015)

1.3.1.1 Cognitive impairment in Parkinson's disease

Cognitive decline is one of the most common PD-related non-motor symptoms and it contributes in a significant manner to morbidity and mortality (Aarsland *et al.*, 2017; Hanagasi *et al.*, 2017). In addition, cognitive impairment has acquired an increasing clinical interest because of its heterogeneous manifestation, and the risk of evolving in dementia in a large number of PD patients (Biundo *et al.*, 2016). Cognitive deficiencies in PD affects a

variety of domains and it generally worsens with disease progression leading to a clinical status known as PDD. Dementia stands for a sufficient level of cognitive impairment that results in a significant reduction in the ability to perform normal daily activities. As dementia in PD accounts for high public health cost and is a frequent cause of patient institutionalisation, it is an urgent issue to cope with (Emre, 2003; Biundo *et al.*, 2016; Hanagasi *et al.*, 2017; Aarsland *et al.*, 2017).

As mentioned above, the clinical manifestation of cognitive deficits in PD are numerous and involves several domains. Executive functions such as the ability to plan activities, the organisation and the regulation of specific behaviour aimed at a specific goal are generally impaired (Emre *et al.*, 2003). In addition, PD patients are defective in their working memory, in memory recall and in verbal fluency. Furthermore, patients experience attention problems with vigilance reduction and fluctuating levels of alertness (Biundo *et al.*, 2016). Another affected domain includes the visuospatial and the perceptive ability. In fact, PD patients report difficulties in the perception of the extra personal space and in the recognition of objects from their shape (Biundo *et al.*, 2016; Aarsland *et al.*, 2017).

Based on the heterogeneity of cognitive deficits it is possible to distinguish two independent and partially overlapping syndromes: I) an early stage syndrome characterised by fronto-striatal dysfunction which is accompanied by working memory deficits, as well as attention and planning problems; II) a late stage syndrome involving a more posterior cortical degeneration with loss of cholinergic neurons that may evolve in PDD (Biundo *et al.*, 2016; Hanagasi *et al.*, 2017).

1.3.2 Dementia with Lewy bodies

Beside PDD, a second dementia subtype among α -synucleinopathies is the DLB. Initially described as rare, DLB is now depicted as the second most common type of degenerative dementia in elderly after Alzheimer's disease (AD) (McKeith *et al.*, 2004; McKeith *et al.*,

2017). The core feature of DLB is the occurrence of dementia that, as previously described, stands for a progressive deterioration of cognitive functions with a magnitude sufficient to interfere with normal social and occupational functions, as well as with usual daily activities (McKeith *et al.*, 2017).

The recognition and the final diagnosis of DLB can be only done through post-mortem assessment of the LB and LN distribution in demented subjects, and the retrospective analysis of their clinical history (Outeiro *et al.*, 2019).

In addition to dementia, a wide spectrum of symptoms occurs in DLB. Fluctuating levels of cognitive impairments with spontaneous alterations in cognition and attention are usual. They may vary over minutes, hours or days and include waxing and waning episodes of inconsistent behaviour, speech and altered consciousness (McKeith *et al.*, 2004; McKeith *et al.*, 2017). Psychiatric manifestations are common and, although they occur in early stage, are persistent during the progression of the pathology (McKeith *et al.*, 2017, Outeiro *et al.*, 2019). DLB patients experience delusions, apathy anxiety and recurrent visual hallucination which are complex and described in 80% of subjects. Visual hallucinations are well-formed, featuring animate figures (people, children and animals) and generally accompanied by sense of presence and visual illusions (McKeith *et al.*, 2004; Outeiro *et al.*, 2019). Of note, although DLB and AD share common clinical manifestations that may overlap and make the correct clinical diagnosis difficult, the occurrence of visual hallucination is more common in the former and should be taken into account for a DLB rather than an AD diagnosis (Outeiro *et al.*, 2019). A further core feature of DLB is the RBD which is characterised by recurrent dream behaviour that includes movements mimicking dream content and is associated with loss of the normal REM sleep atonia (McKeith *et al.*, 2004). Additionally, DLB patients may present parkinsonism, autonomic failures (*e.g.* orthostatic hypotension, urinary incontinence ...) and hypersomnia (McKeith *et al.*, 2017; Outeiro *et al.*, 2019).

1.3.3 Dementia in Parkinson's disease and Dementia with Lewy bodies

As mentioned above, dementia and LB pathology are features shared by two pathologies, PDD and DLB, which are commonly defined with the “umbrella” term Lewy body dementia (LBD) (Sanford, 2018).

Since PDD and DLB share common symptoms, and in later stages they are indistinguishable, an arbitrary “1-year rule” based on the relationship between cognitive decline and motor symptoms has been introduced. While onset of cognitive decline within 12 months of motor symptoms defines DLB, more than 12 months of motor symptoms before cognitive decline identify PDD. In other words, in DLB the cognitive decline precedes or accompanies the first motor symptoms. Conversely, in PDD motor symptoms precede the cognitive decline by at least one year (McKeith *et al.*, 2004; Galasko, 2017).

As previously described, cognitive impairment in both PDD and DLB consists in deficits involving the attention, the visuospatial functions and the executive functions. Patients fail in the evaluation of distance, they show impairment in judgment and become unable to organise and plan activities. On the other hand, in PDD and DLB cognitive deficits are similar across multiple domains. Damages in working memory, long-term memory, visuospatial memory and procedural learning are reported in both PDD and DLB (Emre, 2003; Jellinger 2018). Moreover, memory impairment generally worsens with progression in both pathologies (Galasko, 2017; Jellinger, 2018).

PDD and DLB patients experience complex hallucinations that involve the visual system, and that they can remember in detail. Fluctuation in attention or alertness is also a common feature, but it is more frequently reported in DLB than PDD patients (Galasko, 2017; Jellinger *et al.*, 2018).

Loss of smell and constipation can be found in both DLB and PDD, and they may precede the onset of cognitive and motor symptoms.

For a comprehensive view of overlapping and discriminating clinical features between DLB and PDD refer to Table 1, where motor and cognitive aspects of the pathologies are described.

The overlap between DLB and PDD includes more than LB pathology and symptoms. Both DLB and PDD have indeed synaptic dysfunction in the hippocampus and the typical brain lesions associated to AD (amyloid- β deposits and neurofibrillary tangles) (Jellinger, 2018). Although the AD pathology is commonly observable in DLB and PDD, the load of amyloid- β deposits is significantly higher in DLB than PDD patients (Hepp *et al.*, 2016). Moreover, the load of α -syn lesions in the CA2 region of the hippocampus and in the entorhinal cortex is higher in DLB than PDD (Jellinger, 2014). These significant differences may account for the different severity and progression of dementia, and they point out the possible synergistic activity of LBs and AD pathology in cognitive impairment (Jellinger, 2018).

<i>Overlap</i>	<i>Dissimilarities</i>
Rigidity, akinesia	Some cognitive dysfunctions: deficiencies of attention greater, episodic verbal memory tasks lower in DLB
Cognitive impairments	Tremor significantly less frequent in DLB
Frontal executive dysfunction	Hallucinations more frequent in DLB
Visual-constructive impairment	Onset of dementia later in PDD
Mild language impairment	Orthostatic hypotension more frequent in DLB
Visual hallucinations	Frontal/temporal-associated cognitive subsets more severe in DLB, cognitive decline is faster in DLB than in PDD
Delusions (less frequent)	Delusions, attentional fluctuation and visual hallucinations more frequent in DLB
Depression, anxiety	Visual hallucinations: spontaneous in DLB; after L-dopa therapy in PDD, but also in drug-naïve patients
RBD	
Neuroleptic sensitivity	

Table 1. Clinical overlap and dissimilarities between DLB and PPD patients. Modified from Jellinger, 2018

1.4 The Braak staging of Parkinson's disease

In 2003 Braak and colleagues proposed a unifying staging scheme to determine the progression and severity of PD. The theory implies that the Lewy pathology spreads with a specific pattern in anatomically connected brain regions and it is supported by the evidence of cell-to-cell transfer of misfolded α -syn (Jang *et al.*, 2010; Halliday *et al.*, 2011).

The Braak's staging of PD counts six stages (Figure 6). In stage I and II, LBs and LNs affect the olfactory region, the dorsal motor nucleus of the *vagus* and the *locus ceruleus*. These stages are characterised by non-motor symptoms that involve autonomic and olfactory functions. Stages III and IV have Lewy inclusions in the SNpc, in the trans-entorhinal cortex and in the CA2 region of the hippocampus. Patients at this stage display classical motor symptoms. Stages V and VI with depositions in cortical association areas such as temporal, insular and anterior cingulate cortices and a progression in the entire neocortex. Clinically, these stages account for the emotional and cognitive disfunctions (Braak *et al.*, 2003).

Although the Braak's staging has been initially accepted with enthusiasm, several lines of evidence suggest that this staging scheme cannot be always applied. In fact, there are three different PD phenotypes which differ in timing, age of symptoms onset, and severity of cognitive impairment. These observations indicate that the distribution of LBs and LNs are not sufficient to explain such a diverse scenario like PD clinical features (Halliday *et al.*, 2011).

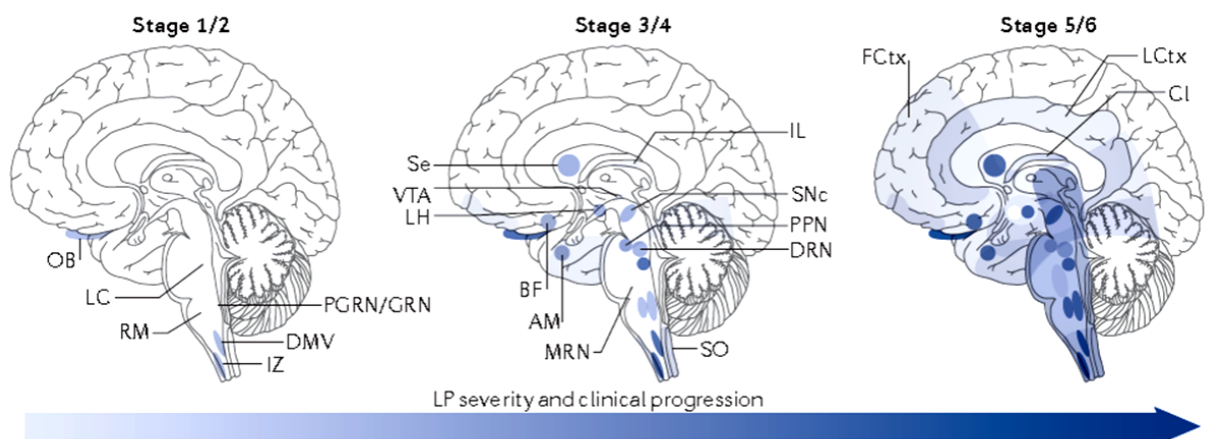


Figure 6. Schematic representation of the Braak staging of PD. Spreading of the Lewy pathology within different brain structures, based on the study of Braak. The progression of disease through the different brain regions increases over time (from left to right) with a progressive increase in the Lewy pathology. Abbreviation: **AM**, amygdala; **BF**, magnocellular nuclei of the basal forebrain; **BNST**, bed nucleus of the stria terminalis; **CI**, claustrum; **cp**, cerebral peduncle; **DMV**, dorsal motor nucleus of the vagus; **DRN**, dorsal raphe nucleus; **FCtx**, frontal cortex; **GP**, globus pallidus; **GPe**, GP externa; **GPI**, GP interna; **HN**, hypoglossal nucleus; **IC**, internal capsule; **ICP**, inferior cerebellar peduncle; **IL**, intralaminar nuclei of the thalamus; **ion**, inferior olivary nucleus; **IZ**, intermediate reticular zone; **LC**, locus coeruleus and subcoeruleus; **LCtx**, limbic cortex; **LH**, lateral hypothalamus; **mcp**, middle cerebellar peduncle; **MRN**, median raphe nucleus; **OB**, olfactory bulb; **opt**, optic tract; **OT**, olfactory tubercle; **PAG**, periaqueductal grey; **PBN**, parabrachial nucleus; **PGRN/GRN**, paragigantocellular and gigantocellular reticular nucleus; **PPN**, pedunculopontine nucleus; **PRN**, pontine reticular nucleus; **pt**, pyramidal tract; **RM**, raphe magnus; **RRF/A8**, retrorubral fields/A8 dopaminergic cell group; **SC**, superior colliculus; **Se**, septum; **SNc**, substantia nigra pars compacta; **SNr**, substantia nigra pars reticulata; **SO**, solitary tract nuclei; **STN**, subthalamic nucleus; **Str**, striatum; **SVN**, spinal vestibular nucleus; **T**, thalamus; **VTa**, ventral tegmental area; **ZI**, zona incerta. From Surmeier *et al.*, 2017

1.4.1 The controversial role of Lewy Bodies

Despite LBs are considered being the histopathological hallmarks of PD, PDD, and DLB, their role in neuronal cell death has long been a matter of intense debate. Evidence supporting the cytotoxicity of LBs stems from observations including: I) neuronal loss peaks in those regions characterised by stronger accumulation of LBs; II) in patients with a mild pathology the number of neurons with LBs is higher than those found in patients with severe pathology, implying that inclusion-containing cells are dying neurons; III) LB density at the cortex might be one of the major correlates of cognitive impairment in PD and DLB; IV) LBs seem to affect neuro-vesicle transport (Wakabayashi *et al.*, 2013).

In stark contrast, other findings suggest that LBs might arise as a secondary effect of the pathology with marginal, if at all, involvement in neuronal loss. In fact, it has been shown that neurons in the SNpc undergo apoptotic-like cell death regardless of whether or not they contain LBs (Tompkins *et al* 1997; Milber *et al*, 2012). Furthermore, LBs have been found

in healthy individuals and the LB load poorly correlates with the severity of symptoms such as cognitive impairment and dementia (Parkkinen *et al.*, 2005; Parkkinen *et al.*, 2008; Frigerio *et al.*, 2011). Alternatively, LBs could represent a protective mechanism to segregate and enhance the degradation and clearance of damaged and cytotoxic proteins (Olanow *et al.*, 2004; Chartier and Duyckaerts, 2018).

The controversial role of LBs has been partially overcome after the identification of insoluble and fibrillar α -syn as the main constituent of LBs (Spillantini *et al.*, 1998). Specifically, studies on the different species of α -syn aggregates found the small soluble aggregates being more harmful than the larger ones (Winner *et al.*, 2011). A detailed description of α -syn biology is provided in chapter II.

1.5 Epidemiology of Parkinson's disease

PD is generally a late onset pathology that affects 1% of worldwide population above 60 years of age. However, early onset PD cases may occur before 40 years old and it represents less than 5% of whole cases (Tysnes and Storstein, 2017). In light of the aetiology, PD can be distinguished in a familial/genetic or an idiopathic/sporadic form. The former is associated with genome alterations which frequently trigger to autosomal dominant or recessive inheritance of PD. The latter has a not well-defined cause and it may result from the interplay between several factors. Familial PD only represents 5-10% of all PD cases (Tysnes and Storstein, 2017).

Although PD affects both genders, it is slightly more frequent in males than females with a male-to-female ratio of approximately 3 to 2 (Kalia and Lang, 2015; Bhat *et al.*, 2018).

The prevalence of PD is higher in Europe, North America and South America compared with Asian and African countries. The most widely accepted rate of prevalence in general population approximates to 100-200 cases for 100000 people. The global incidence ranges from 10 to 18 new cases for 100000 person-years (Kalia and Lang, 2015; Tysnes and

Storstein, 2017). Interestingly, PD incidence may be higher than the reported data. This concern comes from the possibility that incidence studies may be biased by the under-diagnosing of PD, particularly in early onset PD cases or during early disease stages.

Age is considered the main risk factor for developing PD. In fact, both prevalence and incidence increase with the aging of the general populations, where they peak after 80 years old (Figure 7) (Driver *et al.*, 2009; Pringsheim *et al.*, 2014). The drastic increase in prevalence after 80 years age cannot be linked with a comparable growth in incidence rate, but it can be ascribed to health care improvement (Poewe *et al.*, 2017).

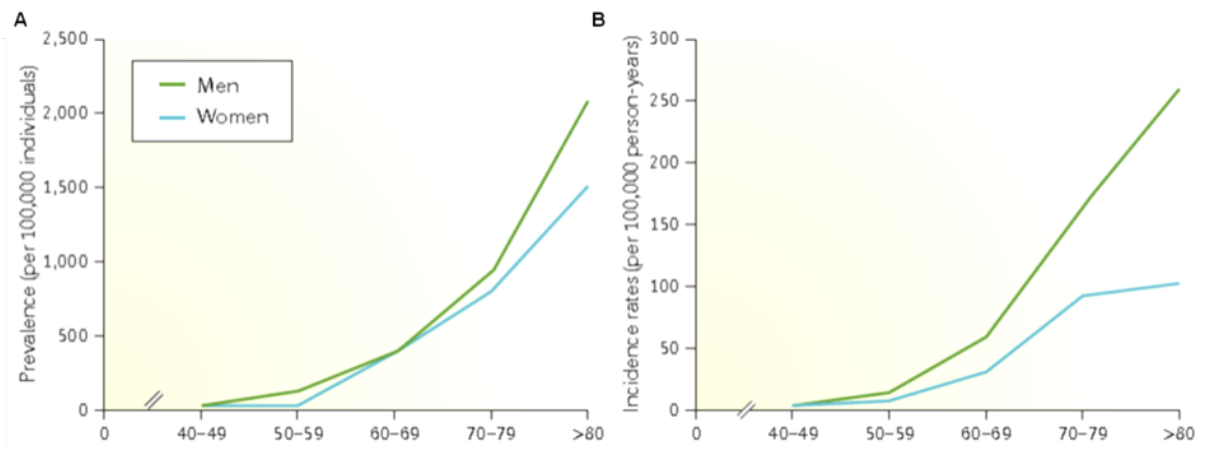


Figure 7. Prevalence (A) and incidence (B) of PD in male and female. The graphs show the increase in incidence and prevalence of PD for 100000 persons in both men (green line) and women (blue line). Modified from Poewe *et al.*, 2017.

The worldwide increase in life expectancy and the aging of general population account for a significant increase in the frequency of neurodegenerative disorders. The number of PD patients is estimated to double in the next 10 years (Dorsey *et al.*, 2007), bearing important implications on the management of the public health care.

In PD patients the mortality does not increase within the first decade after disease onset, but it increases significantly thereafter with the pathology progression compared with global healthy control population (Poewe *et al.*, 2017).

1.5.1 Epidemiology of Lewy body dementia

The lack of well-defined criteria to clinically diagnose LBD makes the assessment of prevalence and incidence of the disease tough. Nevertheless, it is estimated that the prevalence of LBD in people aged 65 years is 1% (0.7 % affected by DLB and 0.3 % by PDD) and, it is widely accepted that LBD represents the second most common neurodegenerative dementia subtype after AD (McKeith *et al.*, 2004; Sanford, 2018). In a study performed from 1991 to 2005 on 542 cases of incident parkinsonism, the reported incidence for DLB is 3.5 and for PDD 2.5 out of 100000 person-years (Savica *et al.*, 2013). Moreover, cognitive impairment in PD generally occurs in advanced age and it appears in 80% of patients during the course of the pathology (Hanagasi *et al.*, 2017; Sanford, 2018). The risk to develop DLB seems to be higher in males than females, whereas not remarkable differences emerge among genders in PDD. 70% of DLB and 49% of PDD are indeed males. Disease onset is a further difference between DLB and PDD, with the former happening earlier (77 years of age) than the latter (82 years of age) (Sanford, 2018).

1.6 Risk factors for Parkinson's disease

Since its first description by James Parkinson, PD has long been considered an environmental dependent pathology. However, it is now well accepted that PD is a multifactorial disorder that originates from the complex interplay between modifiable and non-modifiable risk factors. In particular, the former includes lifestyle and environment, the latter counts of age and genetic predispositions.

1.6.1 Modifiable risk factors

The study of environmental factors triggering to an increased risk to develop PD is difficult and elaborated. This complexity comes from the fact that environmental factors are generally ubiquitous, and this wide distribution makes difficult the identification of control cases not

exposed to the studied stressors. In addition, more than a single factor may be present at the same time and they may act synergistically to increase the risk of developing the pathology. Although the study of environmental risk factors for PD is not trivial, researchers have identified several key elements underlying and contributing to the pathogenesis of both sporadic/idiopathic and genetic PD (Cuenca *et al.*, 2019).

For instance, an increased risk of PD is associated with the exposure to pesticides such as paraquat and rotenone which lead to mitochondrial damage and oxidative stress (Tanner *et al.*, 2011; Kalia and Lang, 2015). In particular, Paraquat has a similar chemical structure of the pro-parkinsonian molecule deriving from the metabolism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP has been the first chemical compound linked to an increased risk of PD and its discovery triggers the investigation of the role of environmental chemicals in PD pathogenesis (Kalia and Lang, 2015; Ascherio and Schwarzschild, 2016).

Modifiable factors positively correlated to the increased risk of PD include rural life, consumption of dairy products, well-water drinking, use of amphetamine and methamphetamine, prior head injury and systemic pathologies (Kalia and Lang, 2015; Ascherio and Schwarzschild, 2016; McKenzie *et al.*, 2017; Cuenca *et al.*, 2019). Intriguingly, brain injury and some systemic pathologies, including diabetes, trigger a long-lasting neuroinflammatory response that has been correlated with an increased risk of developing PD (Ascherio and Schwarzschild, 2016; McKenzie *et al.*, 2017). Despite being a common feature of numerous neurodegenerative disorders, neuroinflammation is not just a mere consequence of the disorder, but it may instead be a pivotal factor in PD pathogenesis and the linker between genetic predisposition and environmental factors (Gao *et al.*, 2011; Kalia and Lang, 2015). In this regard, it has been demonstrated that induction of neuroinflammation, through the intraperitoneal injection of lipopolysaccharide (LPS), in a transgenic PD mouse model leads to an increased loss of SNpc dopaminergic neurons (Gao *et al.*, 2011). On the same line, it has been reported that striatal administration of LPS in

wild-type (WT) mice leads to neuroinflammation and progressive dopaminergic neurodegeneration in SNpc (Choi *et al.*, 2009). On the other hand, several lines of evidence indicate that neuroinflammation may represent a suitable therapeutic target to prevent or delay PD. As a matter of fact, it has been reported that the consumption of non-steroidal anti-inflammatory drugs (NSAIDs) is a protective factor against PD. Among NSAIDs, Ibuprofen has shown the highest protective effect. Moreover, the discordance in the protective effect of Ibuprofen compared with other NSAIDs, such as Aspirin, suggests that Ibuprofen acts via a specific mechanism that may not be shared among other NSAIDs (Hirsch *et al.*, 2012; Ascherio and Schwarzschild, 2016).

The role of neuroinflammation in PD will be further detailed in chapter III.

1.6.2 Non-modifiable risk factors

Age is considered the greatest risk factor for PD. In fact, several data from epidemiological studies clearly indicate an exponential rise of PD incidence and prevalence with advancing age (Kalia and Lang, 2015; Ascherio and Schwarzschild, 2016; Poewe *et al.*, 2017). The biological mechanisms underlying the relationship between aging and PD are still largely unknown (Calabrese *et al.*, 2018). It has been reported that aging may represent a pre-PD state. In this light, aging creates a vulnerable state that approaches the biological threshold required for PD pathogenesis (Collier *et al.*, 2017). In such a scenario, inflammation has been identified as a key player. Indeed, aging is characterised by a progressive inflammatory state, also known as “inflammaging”, in which an equilibrium between pro- and anti-inflammatory signals allows the mitigation of tissue degenerative processes. In PD the normal occurring inflammaging process is deregulated and lacks the homeostasis between pro- and anti-inflammatory signals with a progressive increase in levels of the pro-inflammatory ones. The long-lasting increase in pro-inflammatory mediators may result in

a deleterious microenvironment which in turn could trigger neurodegenerative processes typical of PD (Calabrese *et al.*, 2018).

Genetic contribution is a further component among non-modifiable risk factors. The relevance of genetics in PD comes from the evidence that patients with a familial history of PD have an increased risk to develop the pathology (Kalia and Lang, 2015). The investigation of genetics in PD started in 1997 when Polymeropoulos and colleagues found the first mutation in the gene coding for α -syn (SNCA) linked to PD (Polymeropoulos *et al.*, 1997). As of 1997, mutations in several genes have been identified as causative for monogenic form of PD and, although genetic forms of PD account for only 5-10% of all cases, their investigation has shed new light into pathological mechanisms and molecular pathways which underlie the disease development and progression (Poewe *et al.*, 2017). Genetic forms of PD are responsible for four different kinds of inheritance. Indeed, there are genes that when mutated lead to autosomal dominant, autosomal recessive, X-linked or unclear inheritance forms of PD (Cuenca *et al.*, 2019). Since alterations causing X-linked and unclear inheritance forms of PD are not well understood, except for the fact that they cause a late onset PD, I will only focus on some of those involved in autosomal dominant and recessive PD.

1.6.2.1 Genes involved in autosomal dominant forms of PD

Affected genes causing autosomal dominant PD include SNCA, LRRK2 and VPS35 (Hernandez *et al.*, 2016).

- **SNCA** is the first gene been linked to autosomal dominant inheritance PD. The gene is located on chromosome 4 and codes for a small protein of 140 aminoacidic residues (~14 kDa) α -syn (Spillantini *et al.*, 1995; Lashuel *et al.*, 2013). In familial forms of PD, six missense mutations which cause a single aminoacidic substitution have been reported: A53T, A30P, E46K, H50Q, G51D and A53E (Polymeropoulos

et al., 1997; Krüger *et al.*, 1998; Zarranz *et al.*, 2004; Proukakis *et al.* 2013; Kiely *et al.* 2013; Lesage *et al.*, 2013; Pasanen *et al.*, 2014). Missense mutations in SNCA lead to an early onset PD, and among them A53T is the most frequent (Hernandez *et al.*, 2016). As mentioned above, the missense mutation A53T has been the first PD-linked alteration identified in SNCA (Polymeropoulos *et al.*, 1997). In the same year, Spillantini and co-workers (Spillantini *et al.*, 1997) reported that α -syn was the major constituent of LBs in sporadic PD and in DLB. These distinct findings claimed the relevance of α -syn in PD pathogenesis for the first time, and they strongly suggested that common pathological molecular mechanisms may underlie the development of both sporadic and familial PD. In addition to missense mutations, reassembly of SNCA has also been found in PD. In particular, multiplication of the entire gene has been reported (Singleton *et al.*, 2003; Chartier-Harlin *et al.*, 2004; Ibáñez *et al.*, 2004). A gene dose-dependent effect of SNCA multiplication on PD phenotype has been argued. This hypothesis stems from the evidence that SNCA multiplication, duplication or triplication, leads to a form of PD with onset, progression and severity depending on the SNCA copy number (Figure 8) (Farrer, 2006; Hernandez *et al.*, 2016).

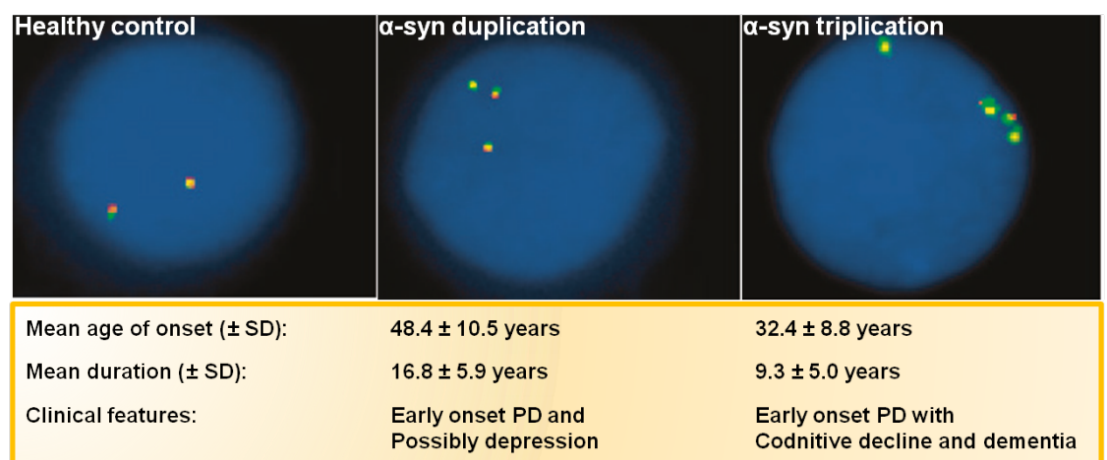


Figure 8. SNCA copy number is linked to PD severity. Fluorescent in-situ hybridisation of SNCA. The images show nuclei containing a different number of SNCA copies (two,

three and four copies) in a normal diploid control (left panel), and in patients with SNCA duplication (middle panel) or triplication (right panel). Nuclear DNA is labelled in blue; SNCA is double labelled in red and green (Overlap between the probes appears as yellow). The increase number of SNCA copy is linked to a worsening in PD severity as shown in the table (Modified from Farrer. 2006).

Intriguingly, alterations in SNCA are also associated with an increase risk to develop both PDD and DLB (Sironi *et al.*, 2010; Schulz-Schaeffer, 2010; Galasko *et al.*, 2017; Jellinger *et al.*, 2018). Although DLB is a relatively common disorder its distribution displays a little aggregation in families, thus making difficult to identify specific DLB risk factors (Outeiro *et al.*, 2019). However, since PD, PDD and DLB share common neuropathological and genetic features, the investigation of the α -syn pathological role in these pathologies, also known as α -synucleinopathies, has gained strong interest over the last few years.

- **LRRK2** is located on chromosome 12 and encodes for a big multidomain protein (Leucine reach repeat kinase 2) of 2527 aminoacidic residues (Li *et al.*, 2014). Variants in LRRK2 are recognised as the most common and frequent root cause of familial PD (Billingsley *et al.*, 2018). More than 100 missense mutations have been reported in LRRK2 and they may be found in 10% of patients with an autosomal dominant inheritance and in 3.6% of patients with sporadic PD (Hernandez *et al.*, 2016; Lesage *et al.*, 2006; Lesage *et al.*, 2009). Among the many LRRK2 variants known, G2019S is the most common pathogenic variant, and it has been found in approximately 4% of patients with a family history of PD (Billingsley *et al.*, 2018). LRRK2 has been implicated in autophagy, cytoskeletal dynamics, kinase cascade, mitochondrial functions and inflammatory reactions (MacLeod *et al.*, 2006; Gloeckner *et al.*, 2009; Parisiadou *et al.*, 2009; Mortiboys *et al.*, 2010; Orenstein *et al.*, 2013; Li *et al.*, 2014; Civiero *et al.*, 2018). Since LRRK2 is involved in a large amount of cellular processes, a clear and univocal pathological mechanism for this

protein in PD is still missing and under investigation. Nevertheless, PD patients carrying LRRK2 variants show a LBs pathology like PD patients carrying SNCA variants. Therefore, it seems that common cellular pathways underlying α -syn aggregation are shared among different genetic PD variants (Spatola and Wider, 2014).

Clinically, patients with a LRRK2-linked PD show a late onset and slow progressive symptoms (Hernandez *et al.*, 2016). Although PD is generally more frequent in males, it has been reported that female patients with the variant G2019S develop PD approximately 10 years earlier than men (Li *et al.*, 2014).

Autosomal dominant PD has also been linked to variants in VPS35 (vesicular protein sorting 35) and EIF4G1 (eukaryotic translation initiation factor 4-gamma 1). The former is involved in the endosomal-lysosomal trafficking complex and its variants are only linked to a slight number of PD cases. Indeed, approximately 0.1% of PD patients carry VPS35 variants (Spatola and Wider, 2014). The latter is involved in mRNA translation processes and accounts for 0.02-0.2% of all PD cases (Spatola and Wider, 2014).

1.6.2.2 Genes involved in autosomal recessive forms of PD

Genes involved in autosomal recessive forms of PD are PARK2, PINK1 and DJ-1. Among these, PARK2 variants are the most commonly observed in PD population (Spatola and Wider, 2014).

- **PARK2** (Parkin) is a 465 aminoacidic residues ubiquitin ligase protein involved in the control of mitochondrial functions. While in healthy condition PARK2 has a cytosolic localisation, in presence of mitochondrial damage the protein translocates to the mitochondrial surface and ubiquitinates membrane proteins leading to the mitophagy process (Hernandez *et al.*, 2016). PARK2 has been the first gene linked to autosomal recessive forms of PD (Kitada *et al.*, 1998). To date, more than 100

variants are known. Both point mutations and exons re-arrangement, deletion and duplication involving all 12 exons of the gene have been reported (Foroud *et al.*, 2003; Hedrich *et al.*, 2004; Lesage *et al.*, 2007). Parkin mutations are linked to early onset PD, and they account for 50% of familial early onset autosomal recessive cases. However, variants in PARK2 are also described in 77% of sporadic PD with an onset before 20 years (Lucking *et al.*, 2000; Spatola and Wider, 2014). Of note, in PARK2-linked PD the presence of LB pathology is not reported (Mori *et al.*, 2003). While an explanation for this is still a matter of debate, it has been speculated that the absence of LB pathology is due to the very early onset of disease. In fact, post-mortem tissues of PARK2 linked PD cases with an older age of disease onset present the characteristic LB pathology of PD (Hernandez *et al.*, 2016).

- **PINK1** has been the second gene identified in autosomal recessive PD cases (Valente *et al.*, 2001; Valente *et al.*, 2004). PINK1 encodes for a 581 aminoacidic residues Ser/Thr kinase (phosphatase tensin homologous-induced kinase 1) with a mitochondrial localisation and, like Parkin, with a role into the mitophagy pathway (Valente *et al.*, 2004; Pickrell and Youle, 2015). Since the discovery of PINK1, a growing number of variants in the gene have been linked to early onset PD, and these account for the second most common cause of autosomal recessive PD (Gandhi and Plun-Favreau 2017)
- **DJ-1** is the third gene identified in autosomal recessive PD, where both missense mutations and exon deletion have been found (Bonifati *et al.*, 2003). DJ-1 related PD has comparable phenotypic features to PINK1 and PARK2-related PD. In fact, PD linked to DJ-1 variants is characterised by an early disease onset (Hernandez *et al.*, 2016). Despite the three genes related to autosomal recessive inheritance PD are associated with an early onset of the pathology, their frequency varies within the PD populations. In particular, DJ-1 variants are rare and they account for less than 1%

of early onset PD cases (Pankratz *et al.*, 2006). DJ-1 encodes for a 189 aminoacidic residues protein with a mitochondrial matrix and inter-membrane space localisation, in addition to its cytosolic expression (Zhang *et al.*, 2005). Variants in DJ-1, like PARK2 and PINK1, are associated with a loss of the protein physiological activities (Hernandez *et al.*, 2016). DJ-1 has been implicated in several protective cellular processes that, when altered, may be involved in PD pathogenesis. Indeed, DJ-1 may serve as a redox sensory molecular chaperone, but also as a transcriptional regulator of anti-apoptotic genes (Menzies *et al.*, 2005; Xu *et al.*, 2005).

Recessive inherited forms of PD are also related to variants in ATPT3A2, PLA2G6 and FBXO7. These variants are rarer than the variants described above and are linked to atypical PD symptoms such as dystonia (Spatola and Wider, 2014).

1.7 Diagnosis of Parkinson's disease

PD is not regarded as a single disease and the term may have a different meaning for clinicians and researchers. In fact, some use the term only for idiopathic parkinsonism associated with LBs inclusions in different brain regions, whereas others use the term as a strictly clinical diagnosis and may accept different pathological conditions underlying the syndrome (Tolosa *et al.*, 2006). Although clinical criteria for the diagnosis of PD have been elaborated, the diagnosis of PD remains difficult especially at the early phases of the disease when symptoms of PD overlap with other forms of parkinsonism (Jankovic, 2008). The use of clinical criteria developed by the UK Parkinson's Disease Society Brain Bank as well as those developed by the MDS International Parkinson and Movement Disorder Society, (Table 2) has significantly improved the accuracy of PD diagnosis. However, population-based studies have revealed that 15% of patients with a diagnosis of PD do not fulfil the criteria for the disease and, 20% of patients are not formally diagnosed as PD patients (Tolosa *et al.*, 2006; Poewe *et al.*, 2017). In addition to the prominent motor symptoms, PD

patients may manifest a wide range of non-motor features (sleep disorder, reduced olfactory ability, constipation, anxiety, depression and cognitive decline) that should be considered in the diagnostic process (Cuenca *et al.*, 2019). Even though clinical diagnosis of PD, which is based on the manifestation of typical motor features, is improved in its accuracy and sensitivity, the gold standard for PD confirmation remains the neuropathological examination of post-mortem brain samples. In particular, well accepted and widely used criteria consist in the presence of a moderate or severe loss of dopaminergic neurons in SNpc with LBs in the surviving neurons, and the absence of evidence for other diseases that may result in parkinsonism (Kalia and Lang, 2015).

MDS diagnostic criteria for PD

Step 1: diagnosis of parkinsonism (core feature)

Presence of bradykinesia as a slowness of movement and a decrement in amplitude or speed (or progressive hesitations or halts) as movements are continued. In combination with at least one of: rigidity and/or rest tremor.

Step 2: determining PD as the cause of parkinsonism with two levels of diagnostic certainty

Diagnosis of clinically established PD requires all three of the below parameters:

Absence of absolute exclusion criteria. These criteria include clinical or imaging evidence for alternative diagnoses of parkinsonism, such as atypical parkinsonism, drug-induced parkinsonism or essential tremor.

Two or more supportive criteria. These include L-DOPA responsiveness, the presence of classic rest tremor, the presence of L-DOPA-induced dyskinesias, the presence of either olfactory loss or cardiac sympathetic denervation on metaiodobenzylguanidine (MIBG) scintigraphy.

No red flags. This refers to features that are unusual but not absolutely exclusionary for PD, for example, the rapid progression of gait impairment that requires wheelchair use or the development of severe autonomic failure within 5 years after onset.

Diagnosis of clinically probable PD requires:

Absence of absolute exclusion criteria (mentioned above)

Presence of red flags (mentioned above) that are counterbalanced by supportive criteria

Table 2. MDS guidelines for the diagnosis of PD. Modified from Poewe *et al.*, 2017.

As mentioned above the diagnosis of PD is difficult. To further improve the diagnostic accuracy clinicians often use further diagnostic tests, and researchers are working at the identification of biological markers that may be useful for an early diagnosis (Poewe *et al.*,

2017). Among the additional tests that clinicians may adopt there are genetic tests and neuroimaging.

Five to ten percent of PD cases have a genetic root cause. Since genetic related PD only account for a small percentage of PD cases, the genetic testing is not part of the routine diagnostic process. On the other hand, patients with a family history of PD or with an early onset of the pathology, which is generally present in autosomal recessive related PD (Poewe *et al.*, 2017), are genetically investigated.

Neuroimaging became relevant in PD after 1980, when for the first-time striatal dopamine depletion in PD patients was demonstrated through F-labelled L-DOPA and PET. Structural MRI is helpful to identify symptomatic parkinsonism, and it allows to evaluate macroscopic brain changes such as a deterioration of the SN. Furthermore, various MRI techniques (*e.g.* Neuromelanin-sensitive MRI) provide a valuable diagnostic support to reveal specific changes (Poewe *et al.*, 2017; Cuenca *et al.*, 2019).

1.8 Diagnosis of Dementia with Lewy bodies and Dementia associated to Parkinson's disease

The clinical diagnosis of a probable DLB is based on the assessment of at least two of the core features of the disease, whereas the diagnosis of possible DLB is made when only one of the core features is presented (Table 3) (McKeith *et al.*, 2004; Outeiro *et al.*, 2019).

DLB core features include fluctuating levels of cognitive impairments, visual hallucinations, sleep behaviour disorders (*e.g.* RBD, excessive daytime drowsiness). In addition, extrapyramidal motor feature may occur.

DLB and PDD largely overlap in their clinical manifestations. A possible differentiation between the two pathologies may result from the assessment of extrapyramidal motor features. Indeed, DLB patients are characterised by an absence or a moderate grade of extrapyramidal motor features, at least until late stages. On the other hand, PDD patients

show prominent motor features, while cognitive impairments generally occur later (Outeiro *et al.*, 2019).

Revised criteria for the clinical diagnosis of probable and possible DLB

Essential for a diagnosis of DLB is dementia, a progressive cognitive decline of sufficient magnitude to interfere with normal social or occupational functions, or with daily activities. Prominent or persistent memory impairment may not necessarily occur in early stages but is usually evident with progression. Deficits on attention, executive function and visuo-perceptual ability may be prominent and occur early.

Core clinical features (Two core features are essential for the diagnosis of probable DLB, one for the diagnosis of possible DLB)

Fluctuating cognition with pronounced variations in attention and alertness.

Recurrent visual hallucinations.

RBD which may precede cognitive decline.

One or more cardinal feature of parkinsonism – these are bradykinesia (defined as slowness of movement and decrement in amplitude or speed), rest tremor, or rigidity.

Supportive clinical features

Severe sensitivity to antipsychotic agents, postural instability, repeated falls, syncope or other transient episodes of unresponsiveness, severe autonomic dysfunction (*e.g.* constipation, orthostatic hypotension, urinary incontinence) hypersomnia, hyposmia, hallucinations in other modalities, apathy, anxiety and depression.

Table 3. MDS guidelines for the diagnosis of PD. Modified from Outeiro *et al.*, 2019.

Of note, post-mortem validation of the clinical diagnosis of DLB is limited, and it is compromised by the lack of defined neuropathological criteria. Autopsy of patients with non-DLB clinical manifestations have clearly shown LB depositions in different brain regions (McKeith *et al.*, 2004). A recent UK study revealed that while only 4.6% of subjects are clinically diagnosed as DLB, LB pathology is presented in 20% of post-mortem brains. This indicates that an underestimation of DLB during life occurs, and that the clinical criteria are not yet sufficiently sensitive and specific. In fact, DLB may present features reminiscent of AD, and therefore misdiagnosed as AD (Outeiro *et al.*, 2019). Generally, while AD patients are characterised by dysfunctions of episodic memory, DLB subjects have attention, executive and visuospatial deficiencies (Park *et al.*, 2011). DLB and AD have somewhat specific patterns of deficits; people with AD often have better visuo-spatial and visuo-constructional skills early in their disease, but poorer short-term memory. Hallucinations and

delusions are prominent, persistent and early features of DLB, while in AD they appear later in the course of the disease and are transient (Stavitsky *et al.*, 2006; Tiraboschi *et al.*, 2006).

Introduction

Chapter II

2.1 The synuclein family: a brief overview

Synuclein has been first identified in 1988 by Maroteaux and colleagues in the electric organ of the electric ray *Torpedo californica*. The protein was named after its cellular localisation in the pre-synaptic terminals (SYNapse) and on the nuclear envelope (NUCLEus) (Maroteaux *et al.*, 1988; Lavedan, 1998). To date, synuclein refers to a family made of three distinct proteins that share common features: α -, β - and γ -synuclein (α -, β - and γ -syn) (Wales *et al.*, 2013). In humans, α -syn was discovered during the investigation of the β -amyloid plaque composition from AD patients. In fact, researchers identified a small peptide known as NAC (non-amyloidogenic component of amyloid plaques) within β -amyloid plaques. The precursor of NAC (NACP), was found to be homologous to the rat α -syn (Ueda *et al.*, 1993), whereas the β -syn was first isolated as a bovine brain-specific phosphoprotein of 14 kDa (phosphoneuroprotein) (Lavedan, 1998). Therefore, NACP and the human ortholog of phosphoneuroprotein were recognised as two distinct synucleins and termed α - and β -syn, respectively (Jakes *et al.* 1994).

Synucleins are soluble, heat-resistant and highly conserved proteins in vertebrates. In particular, all three synucleins share an amphipathic N-terminal domain, whereas they essentially differ in their acidic C-terminus (Figure 9) (George, 2002).

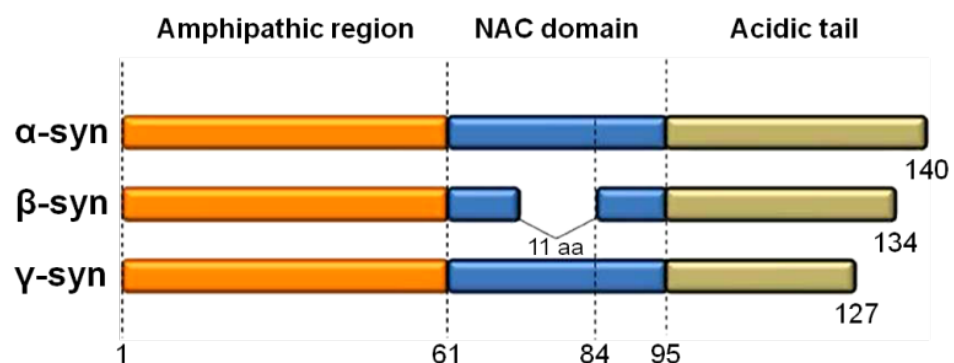


Figure 9. Schematic representation of the α -, β -, and γ - synuclein isoforms. Synuclein isoforms consists of three domains. The conserved N-terminal amphipathic region is depicted in orange. In blue is the central hydrophobic NAC domain. In gold, the acidic C-terminal tail, whose length differs within the three forms (Wales *et al.*, 2013).

With 140 aminoacidic residues in its primary structure, α -syn is the longest among synucleins. 134 residues make up β -syn, whereas γ -syn is the shortest one with 127 residues. β -syn is closely comparable with α -syn, made exception for the lack of 11 residues in the NAC domain of β -syn compared to α -syn (Wales *et al.*, 2013). Of note, α -, β - and γ -syn have different expression patterns. While α -syn and β -syn are predominantly expressed in the brain, γ -syn is found in the peripheral nervous system and notably in malignant breast tumours (Jakes *et al.*, 1994; Iwai *et al.*, 1995; Buchman *et al.*, 1998; Ji *et al.*, 1997).

2.2 Alpha-synuclein: a key protein in PD and α -synucleinopathies

In the last twenty years huge efforts have been made to characterise the pathological roles of α -syn in PD, and more generally in a class of pathologies known as α -synucleinopathies. α -synucleinopathies refer to distinct clinical entities (e.g. PD, LBD, MSA) which share common neuropathological lesions (LBs and LNs) mostly consisting of fibrillar and insoluble α -syn (Spillantini *et al.*, 1997; Spillantini *et al.*, 1998; Baba *et al.*, 1998; Wales *et al.*, 2013; Alafuzoff and Hartikainen, 2018).

The interest in studying α -syn in α -synucleinopathies stems from accumulating evidence of the potential harmful role of the protein including: I) missense points mutations in the gene encoding for α -syn in families with hereditary PD and DLB; II) α -syn as the main constituent of LBs and LNs; III) the fact that duplication and triplication of the entire SNCA locus lead to familial PD; IV) genome-wide association studies revealing that genetic variants in the SNCA locus represent a risk factor for sporadic PD; V) animal models overexpressing WT or mutant α -syn showing histopathological and some clinical features of PD (Deleersnijder *et al.*, 2013; Bengoa-Vergniory *et al.*, 2017).

2.3 Insight into α -syn structure and physiological functions

As mentioned above, α -syn is the product of SNCA, a gene located at position 21 on the long arm of chromosome 4 (Figure 10) (Spillantini *et al.*, 1995). α -syn expression and localisation change over time, with its levels increasing through the early weeks of development, while it diffuses from the soma to the pre-synaptic terminals (Burré *et al.*, 2015).

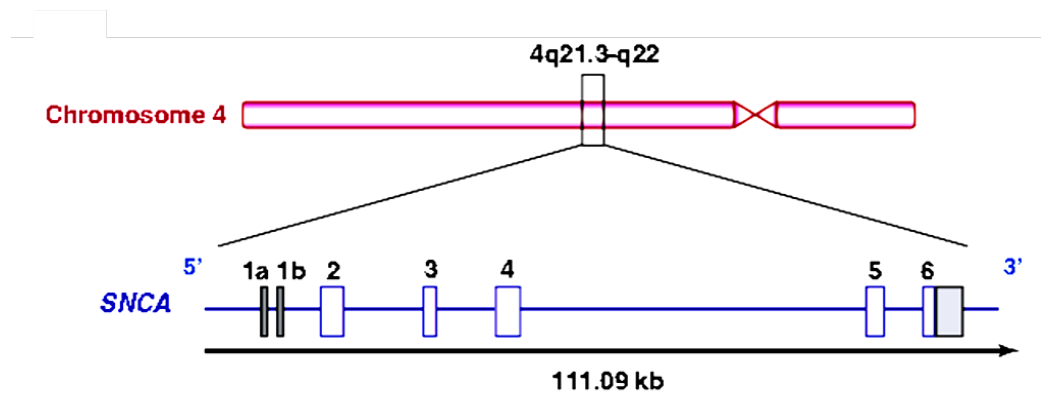


Figure 10. Schematic of the localisation and structure of SNCA. Modified from Venda *et al.*, 2010.

Because α -syn lacks a well-organised secondary structure, it belongs to the intrinsically disordered protein family, and it is generally defined as a disordered or naturally unfolded protein (Deleersnijder *et al.*, 2013; Theillet *et al.*, 2016; Zhang *et al.*, 2018).

Three different domains can be distinguished within the primary structure of α -syn (Figure 11A): I) the N-terminal domain (residues 1-60), II) the central hydrophobic region (residues 61-95), and III) the C-terminal domain rich in prolines and the acidic residues aspartate and glutamate (residues 96-140) (Villar-Piqué *et al.*, 2016). The N-terminal domain is characterised by a series of 11-residues imperfect repeats with a conserved hexameric motif (KTKEGV). While α -syn N-terminus remains unstructured in solution, it adopts an amphipathic α -helical secondary structure in presence of lipids (Figure 11B), which is a typical conformational feature of membrane recognition and binding (Eliezer *et al.* 2001; Deleersnijder *et al.*, 2013; Wales *et al.*, 2013; Zhang *et al.*, 2018).

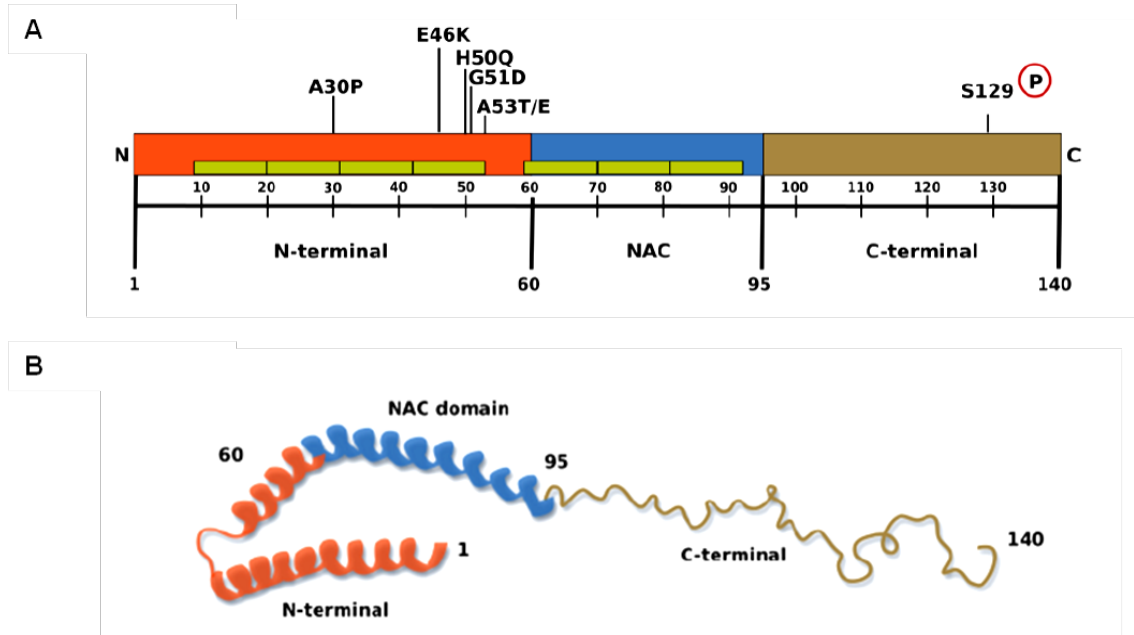


Figure 11. Domain structure of α -syn. (A) Schematic representation of α -syn regions. The N-terminus (residues 1-60) is amphipathic and responsible for the interaction of α -syn with membranes. It includes repeats of the KTKEGV motif (green rectangles). The central region (residues 61-95), termed NAC, is a hydrophobic region and it is involved in the aggregation process. The C-terminus (96-140) is characterised by the presence of acidic residues bearing a negative charge. (B) Schematic cartoon representation of micelle-bound α -syn. The N-terminal region (orange), the NAC region (blue) and the unstructured C-terminus (gold). Modified from Gallegos *et al.*, 2015.

Remarkably, all point mutations associated to familial PD (A53T, A30P, E46K, G51D and H50Q) clusters in the N-terminal domain (Bendor *et al.*, 2013), suggesting that this region has an important function in regulating the α -syn propensity to aggregate, a process with strong implications in α -synucleinopathies pathogenesis (further details in section 2.4).

The central hydrophobic portion of α -syn, also known as the NAC domain, is the second major component of A β -amyloid plaques in AD (Irizarry *et al.*, 1996). It contains two KTKEGV motifs and accounts for the aggregation ability of the protein (Wales *et al.*, 2013; Deleersnijder *et al.*, 2013). Indeed, this portion may undergo a conformational change that generates a β -sheet structure in spite of its native random coil (Belluci *et al.*, 2012).

The acidic C-terminal region domain contains 5 proline, 10 glutamate, and 5 aspartate aminoacidic residues (Deleersnijder *et al.*, 2013; Wales *et al.*, 2013). Thus, because of its

low hydrophobicity and its overall net negative charge, the C-terminal domain is responsible for the naturally unfolded and disordered structure of α -syn (Figure 11B) (Deleersnijder *et al.*, 2013). Of note, some residues within the α -syn C-terminus can be phosphorylated. Among these, Serine in position 129 is found phosphorylated in almost 90% of α -syn inside LBs, although the role of this post-translational modification is poorly understood and still under investigation (Gallegos *et al.*, 2015; Anderson *et al.*, 2006; Samuel *et al.*, 2016).

In conclusion, the secondary structure of α -syn lacks a unique and defined conformation, with the exception of some α -helical structures in its N-terminal portion. Such properties provide α -syn with some conformational flexibility, so that the protein can adopt a wide range of structures depending on the environment and binding partners (Jain *et al.*, 2013). As protein structure and function are intimately linked, the versatile and dynamic structure of α -syn results in the multifunctional properties of the protein (Lashuel *et al.*, 2013). Besides its unfolded nature, several challenges make the understanding of α -syn functions difficult: I) α -syn overexpression leads to neurotoxicity, and II) the complete depletion of α -syn does not lead to significant alterations but only causes an altered synaptic transmission, which may be compensated by the activity of other proteins (Burré *et al.*, 2015; Villar-Piqué *et al.*, 2016). As a result, despite numerous hypothetical functions have been put forward and attributed to α -syn, none is fully consensual. Thus, α -syn functions in physiological and pathological conditions still remain controversial.

Among the various functions proposed for α -syn, one of the most widely accepted is its role in pre-synaptic terminals and particularly in the regulation of synaptic transmission. Such functions are suggested by numerous experimental observations. Depletion of α -syn is not lethal and does not lead to a neurodegenerative phenotype, pointing out the possible redundant functions of α -, β - and γ -syn (Lashuel *et al.*, 2013; Wales *et al.*, 2013; Calo *et al.*, 2016; Spillantini and Goedert, 2018). On the other hand, deletion of all the three synucleins

leads to a more severe, although yet viable, phenotype characterised by a deficient SNARE-complex assembly and an increased striatal dopamine release (Wales *et al.*, 2013; Calo *et al.*, 2016). Beside the genetic manipulation of synucleins expression, supporting evidence for a synaptic function of α -syn includes the predominant localisation of the protein into pre-synaptic terminals (Figure 12A, B), and the co-localisation with the vesicle reserve pool of synapses (Lee *et al.*, 2008). Through its C-terminal domain, α -syn directly interacts with VAMP2 and, through a chaperone-like activity, promotes the SNARE complex assembly which is a component of the membrane fusion machinery (Figure 12C) (Burré *et al.*, 2010). SNARE proteins include a large number of integral membrane proteins involved in the docking and fusion between pre-synaptic vesicles and the cell membrane. Two groups of SNARE proteins can be identified based on their localisation: v-SNARE proteins which are located into the pre-synaptic vesicle membranes, and the t-SNARE proteins placed into the pre-synaptic cell membrane.

As α -syn promotes the SNARE complex assembly, its loss of function may trigger changes in neurotransmitter release, synaptic dysfunction and neurodegenerative processes (Burré *et al.*, 2010). Consistently, over-expression of human WT α -syn has been linked to a reduction in vesicle release both in hippocampal neurons and in transgenic (Tg) mice. In this murine model, α -syn over-expression decreased the vesicle recycling pool size and the number of vesicles adjacent to the synaptic active zone. In fact, Tg mice over-expressing human α -syn, displayed a slight increase in the post-synaptic density and a reduction in proteins associated with synaptic vesicles, which may lead to an alteration of synaptic transmission and plasticity, essential physiological processes involved in the formation of new memories (Nemani *et al.*, 2010). In addition, α -syn has been shown to regulate vesicles exocytosis via binding to exocytosis controlling proteins such as phospholipase D2 and the family of small GTPase Rab (Lashuel *et al.*, 2013). These data suggest a possible detrimental role of α -syn,

which in pathological conditions may affect synaptic vesicles recycling (Figure 12C), hence synaptic activity/transmission and cognitive functions.

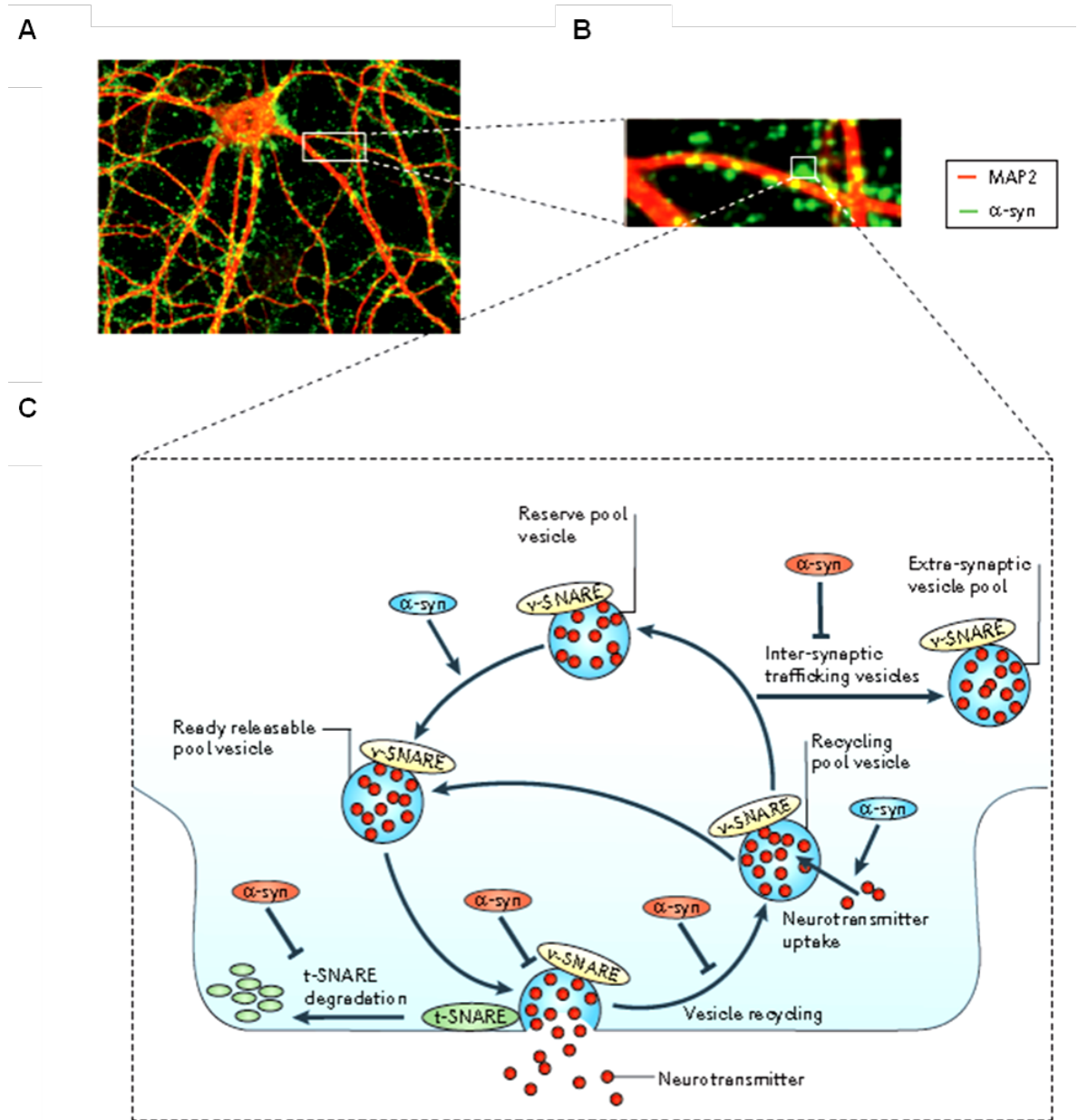


Figure 12. Synaptic localisation and function of α -syn. (A, B) Immunostaining for MAP2 (red) and α -syn (green) reveal the pre-synaptic localisation of α -syn. (C) Schematic representation showing the roles of α -syn at the pre-synaptic terminal in the regulation of vesicle trafficking and vesicle refilling, as well as the interactions between t-SNARE and v-SNARE proteins and neurotransmitter release. Accumulation of α -syn prevents neurotransmitter release, vesicle recycling and trafficking between synaptic buttons and influences the stability of SNARE complex assembly (Lahuel *et al.*, 2013).

As mentioned above, the first synuclein was found in both synaptic terminals and in the nuclear envelope. Nevertheless, the nuclear localisation of α -syn is not largely accepted and it remains controversial with some groups identifying α -syn in the nucleus and some other failing at the same purpose (Li *et al.*, 2002; Yu *et al.*, 2007; Zhang *et al.*, 2008; Huang *et al.*, 2011; Burrè *et al.*, 2018). Although the mechanisms driving the nuclear localisation of α -syn are poorly understood, it seems that the N-terminal domain plays a key role, and PD related missense point mutations in α -syn as well as an increase in oxidative stress appear to increase the protein nuclear translocation (Wales *et al.*, 2013). Transcription regulation is a putative function proposed for α -syn in the nucleus, where two different mechanisms may co-exist. Specifically, α -syn may bind directly the DNA and/or it may influence gene expression by interacting with transcription regulation factors. In this regard, it has been reported that α -syn co-localizes with histone-3 and inhibits its acetylation, leading to an aberrant transcriptional control, which may ultimately result in cell death (Kontopoulos *et al.*, 2006). The transcriptional deregulation caused by α -syn triggers an alteration in the expression of several proteins involved in different signal pathways such as cyclic adenosine monophosphate (cAMP), and response element binding protein (CREB) (Wales *et al.*, 2013). Of note, cAMP and CREB are involved in long term potentiation (LTP), an experimental paradigm measuring synaptic plasticity, and memory formation (Silva *et al.*, 1998). Therefore, alterations in these pathways induced by α -syn may contribute synergistically with many other mechanisms to memory damage.

2.3.1 Memory, synaptic plasticity and LTP

Learning and memory are the result of the interaction of millions of neurons in the brain and their coordinated activity (Stuchlik, 2014).

While memory refers to a capability of virtually any animal to encode, store and retrieve information aimed at guiding behavioural output, learning is viewed as the acquisition and the encoding of information to form new memories (Stuchlik, 2014).

Depending on their duration, memories can be classified into short- and long-term. While short-term memory is the ability to hold and recall information for a short period of time, usually for few seconds, long-term memory stores information for long-lasting periods, essentially for the entire lifetime span. Short-term memory relies on existing networks and post-translational modifications, whereas long-term memory is accompanied by structural and functional changes of neural networks that require *de novo* gene expression (Bisaz *et al.*, 2014)

Long-term memory allows us to store information for long periods of time. Information may be retrieved consciously (explicit memory) or unconsciously (implicit memory). Explicit memory refers to memories that can be consciously recalled such as the knowledge of facts, people and events, whereas the implicit memory represents those memories associated with skills and the ability to perform actions (Camina and Güell, 2017).

From an anatomical/functional standpoint many areas of the brain contribute to the processes of learning and memory, but it has been shown that the hippocampus is the cerebral area that plays a key role in hosting the mechanisms related to memory (Scoville and Milner, 1957).

A crucial point in the field of learning and memory is the concept of neuronal plasticity, which describes structural and functional changes in the brain linked to experience and development. Neuronal plasticity involves events at the synaptic level such as elimination, reinforcement and formation of new synapses.

It is believed that long-term memory is based on the process of synaptic transmission strengthening. Experimental supports to this hypothesis derive from the finding that repetitive activation of excitatory synapses in the hippocampus leads to an increase in synaptic strength that could last for hours and days (Andersen and Lomo, 1966; Bliss and Gardner-Medwin, 1973). This long-lasting synaptic change, known as LTP, has been extensively investigated and it is widely considered as an experimental paradigm for studying synaptic plasticity and molecular mechanisms at the base of learning and memory (Miller and Mayford, 1999; Citri and Malenka, 2008; Stuchlik, 2014). Following an intense stimulation of pre-synaptic neurons, for instance repetitive pre-synaptic tetanic stimulation, the amplitude of the response in post-synaptic neurons increases. In this process two different subtypes of glutamatergic receptors may be activated by the glutamate release: α -amino-3-hydroxy-5-methyl-1-4-isoxazolepropionic receptors (AMPA) and N-methyl-D-aspartate receptors (NMDA). AMPA are channels permeable to monovalent ions, sodium (Na^+) and potassium (K^+), and provide an inward positive current leading to the depolarization of neurons. NMDA, like AMPA, are permeable to ions Na^+ , K^+ , but also calcium (Ca^{2+}) (Baltaci *et al.*, 2019). However, as the pore of NMDA is blocked by the magnesium ion (Mg^{2+}), their activation requires both the binding with glutamate and neuronal depolarization. In fact, during LTP induction, glutamate binds to AMPA leading to a membrane depolarization that may be sufficient to dissociate Mg^{2+} . This process allows the inward flux of Na^+ , K^+ and Ca^{2+} through NMDA causing a drastic increase in the intracellular concentration of these ions, especially in the post-synaptic terminals (Baltaci *et al.*, 2019). Ca^{2+} is an important second messenger. Indeed, Ca^{2+} controls the activity of several enzymes via direct binding or indirectly through regulation of phosphorylation mechanisms. For instance, when four Ca^{2+} ions bind to calmodulin (CaM) the protein becomes active. Activated CaM may in turn activate other enzymes such as adenylate

cyclase and Ca^{2+} /CaM-protein kinase II (CaMKII). The investigation of LTP uncovered two different phases taking place during the process (Baltaci *et al.*, 2019). The early LTP (protein-synthesis-independent) phase starts immediately after LTP induction with a single train of high frequency stimulation, and it produces a brief duration synaptic facilitation by making pre-existing post-synaptic glutamatergic receptors more sensitive to the neurotransmitter (Baltaci *et al.*, 2019). The late LTP (protein-synthesis-dependent) phase, for which a strong stimulation is mandatory, starts hours after the LTP induction. Along with this phase, new protein synthesis occurs, which requires gene transcription. The transcription cascade begins with the transcriptional factor CREB-1, that induces the transcription of genes responsible for the growth of new synaptic connections (Bailey and Kandel., 1993; Baltaci *et al.*, 2019).

2.4 The aggregation of α -syn and the oligomer hypothesis

Protein misfolding and aggregation are shared features of neuropathological disorders such as AD, Prion diseases and α -synucleinopathies. Many research efforts have focused on the understanding of the mechanisms underlying the aggregation process and aimed at identifying the aggregates that are responsible for the diseases.

The aggregation process of α -syn leads to the formation of β -rich fibrillary species from its native unfolded conformation. In pathological conditions α -syn undergoes to nucleation process fostering the formation of soluble oligomers, protofibrils and mature insoluble fibrils (Figure 13), which grow by monomers addition and finally make up LBs and LNs (Buell *et al.*, 2014; Ono, 2017).

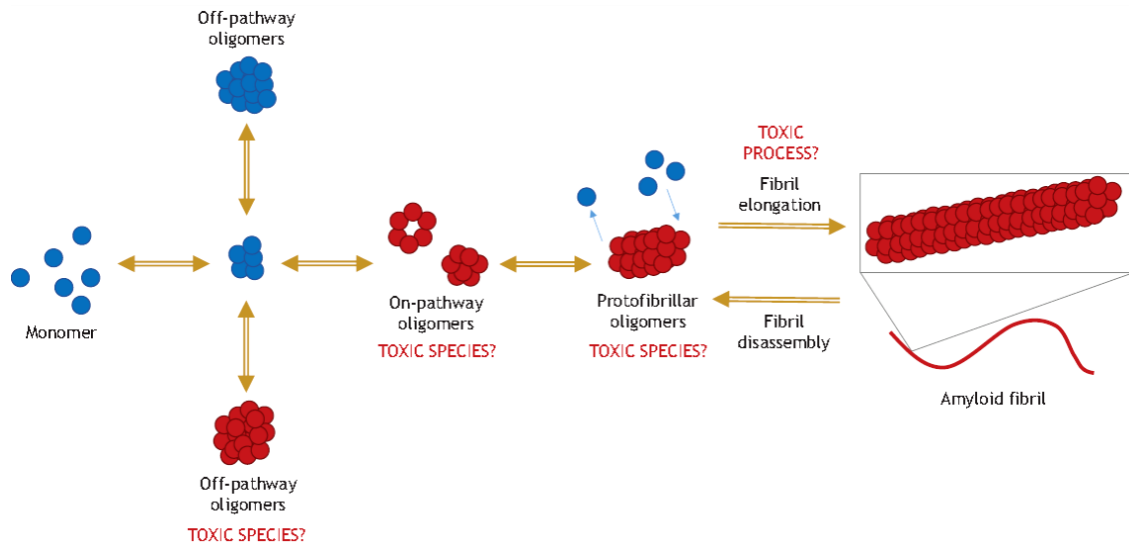


Figure 13. Schematic representation of α -syn aggregation process. Monomeric α -syn may aggregate and form different moieties. Two distinct pathways have been proposed: the off-pathway leads to the production of non-toxic aggregates, the on-pathway generates species which can be neurotoxic (Roberts and Brown, 2015).

As previously described three distinct domains are found in α -syn. Since deletion or disruption of the hydrophobic 12-aminoacid-residue sequence in the central domain abrogates α -syn aggregation (Giasson *et al.*, 2001), the NAC region had been initially identified as crucial for α -syn aggregation. However, deep investigations into α -syn in the recent years additionally claim a relevant role for the N-terminal domain. Of note, all of the point missense mutations related to PD are located within the N-terminus, and they have been shown to influence α -syn aggregation. A53T and E46K variants are widely assumed to increase the aggregation rate of α -syn compared to the WT protein, with A53T being more effective than E46K (Conway *et al.*, 2000; Greenbaum *et al.*, 2005). Intriguingly, either more rapid or slower aggregation kinetics have been reported for the A30P variant (Narhi *et al.*, 1999; Lemkau *et al.*, 2012). H50Q and G51D variants are the most recently identified point mutations linked to PD, and it has been demonstrated that they lead to a divergent effect on the aggregation process. Particularly, H50Q increases the rate of α -syn aggregation, whereas the G51D variant has the opposite effect (Rutherford *et al.*, 2014).

During the aggregation process three different steps may be discerned: the initial formation steps, the growth of the fibrils (elongation), and their amplification (Flagmeier *et al.*, 2016). The single point mutations in α -syn do not affect solely the overall aggregation rate, but they affect in a specific way the distinct steps underlying α -syn aggregation (Flagmeier *et al.*, 2016).

Like the NAC and the N-terminal domain, the C-terminus has also been shown to modulate the aggregation of α -syn. Accordingly, antibodies against this region are able to inhibit the fibrillation of α -syn (Sahin *et al.*, 2017). Taken together, these findings depict a complex context and highlight the need of further investigation aimed at a more punctual and comprehensive understanding of the interplay between the α -syn sub-regions and their impact on its aggregation.

As mentioned above, α -syn oligomers (α -synOs) represent an intermediate aggregate in the process eventually leading to α -syn fibrils. The term “oligomer” is used to describe aggregates without a fibrillar conformation. α -synOs differ in their molecular composition (number of monomers) as well as in their conformation (β -sheets content and hydrophobic region exposure) (Bengoa-Vergniory *et al.*, 2017). Although the mechanisms involved in α -syn toxicity have not been completely elucidated, several data depict α -synOs as the main harmful moiety underlying α -syn deleterious effects. Indeed, α -syn detrimental activities seem to be independent of large α -syn aggregates and more closely associated to the oligomeric species. Exploiting a PD rat model based on the injection of lentiviruses expressing α -syn variants promoting either the oligomer or the fibril formation, Winner and colleagues first demonstrated that loss of dopaminergic neurons in the SN was induced by α -synOs, whereas the expression of the α -syn variant leading to fibrils formation was not effective (Winner *et al.*, 2011). More recently, Cai and co-workers confirmed the role of oligomers in mediating dopaminergic neuronal loss in SN in a new mice model of PD (Cai

et al., 2018). In this work, the SN was injected with adeno-associated viruses carrying a vector encoding the yellow fluorescent protein (YFP) fragmented into its N- or C-terminus separately fused with human WT α -syn to form a non-fluorescent-fusion protein. Through this approach, the authors were then able to show the formation of α -synOs through the observation of the fluorescence deriving from the reconstituted fluorescent protein upon α -syn oligomerization. Furthermore, they found a time-dependent reduction in striatal dopamine levels as well as death of nigral dopaminergic neurons and neuroinflammation (Cai *et al.*, 2018). On the same line, two different groups have found that α -synOs impair LTP in hippocampal brain slices, whereas monomers and fibrils were ineffective (Martin *et al.*, 2012; Diógenes *et al.*, 2012). Together, these findings support the “oligomeric hypothesis” allowing researchers to introduce a new definition of α -synucleinopathies as pathologies related to oligomers, and to classify them under the name of “oligomeropathies” (Forloni *et al.*, 2016; Ono, 2017).

As mentioned above, during the aggregation process several types of α -synOs may be formed, and their toxicity reflects such a heterogeneity. In this regard, it has been shown that different effects on cell survival result upon treatment of neuronal cultures with distinct α -synO species (Danzer *et al.* 2007). Furthermore, the finding of tetrameric α -syn endowed with an α -helical stable conformation in living human cells supports the possible existence of oligomers with a physiological function (Bartels *et al.*, 2011). This hypothesis posits that α -syn tetramers could exert a protective function counteracting the on-pathway of aggregation (the process in which oligomers precede fibrils formation and that is opposed to the off-pathway, where oligomers are stabilized and do not further aggregate). Accordingly, α -syn point mutations may result in the loss of the putative protective property of oligomers (Bartels *et al.*, 2011; Roberts and Brown, 2015).

2.5 Intracellular mechanisms involved in α -synO toxicity

As α -synOs were proposed as pivotal culprits in neurodegeneration, researchers have focused on several cellular targets to explain the potential mechanisms underlying α -synO toxicity. Many intracellular targets (mitochondria, proteasome, endoplasmic reticulum, cell membrane, lysosome and synapse) as well as related processes, seem to be potentially involved in the deleterious activity of α -synOs (Figure 14). However, a complete understanding of α -synO-mediated neurotoxicity is not available yet, and it remains the subject of intensive investigation (Zhang *et al.*, 2018).

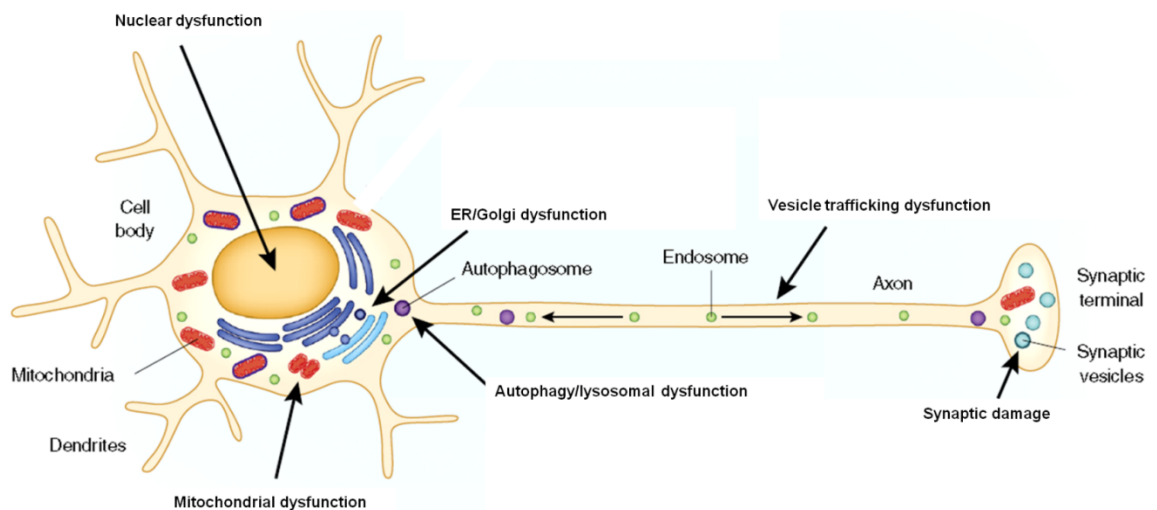


Figure 14. Intracellular targets of α -synOs. Schematic representation of the different cellular targets of α -synOs (modified from Wong and Krainc, 2017).

At the mitochondrial level, α -synOs lead to morphological alterations that in turn result in fragmentation and disruption of the cellular organelle (Plotegher *et al.*, 2014). More recently, treatment of SHSY-5Y cells with α -synOs has been reported to up-regulate the expression of the mitochondrial cytochrome c subunit 2, which leads to the reduction of adenosine three phosphate (ATP) and to an increase in reactive oxygen species (ROS) (Danyu *et al* 2019).

In a PD mouse model and in post-mortem PD samples, α -synOs are shown to accumulate into the endoplasmic reticulum (ER), leading to a chronic ER stress and to a significant damage in protein synthesis and in protein quality (Bengoa-Vergniory *et al.*, 2017).

The ubiquitin-proteasome system is one of the main cellular mechanisms involved in the removal of misfolded and damaged protein. Dysfunction of this protein degradation pathway has been linked to PD pathogenesis. In fact, α -synOs have been reported to interact with the proteasome subunits and to block the proteasome activity (Lindersson *et al.*, 2004; Emmanouilidou *et al.*, 2011). Of note, α -synO-mediated inhibition of the proteasome activity is reverted upon exposure to antibodies that neutralise the interaction or compounds disrupting α -synOs (Xilouri *et al.*, 2013).

Maintenance of cell membrane architecture and permeability properties is crucial for cell survival and functions. Extracellular oligomers of α -syn may insert into membranes generating pore-like structures that could act as non-selective channels, thus resulting in abnormal ionic intake which unbalances intracellular homeostasis (Danzer *et al.*, 2007; Tsigelny *et al.*, 2012). In addition, α -synOs might also interact with the membrane altering the lipid packing. In this regard, Chaudary *et al.* have recently reported that by interacting with damaged cellular membranes, α -synOs may promote the propagation of the membrane defects (Chaudary *et al.*, 2016), therefore opening new investigation routes to elucidate α -synO action mechanisms.

Autophagic and lysosomal loss of function has been linked to PD. Accordingly, α -synO accumulation might result in the autophagic and lysosomal degradation pathway disturbance, which in turn could cause α -syn accumulation and further production of oligomers (Ingelsson, 2016; Ono, 2017; Zhang *et al.*, 2018). The interest in studying the mechanisms of α -synO activity in the context of the lysosomal degradation pathway comes from the evidence that oligomers, and not fibrils, are cleared by lysosomes. Furthermore, blockage of the lysosomal pathway leads to α -synOs accumulation and toxicity (Lee *et al.*, 2004). Hitherto, the most commonly accepted hypothesis indicates a possible vicious circle between α -synOs and the autophagic/lysosomal pathway. Toxic α -synOs may damage

lysosomes leading to an inhibited clearance of α -syn, which in turn causes further production of toxic oligomers (Bourdenx *et al.*, 2014).

Damage and reduction of synapses have been described in PD and PD-related disorders. Notably, altered expression of synaptic proteins involved in synaptic transmission occurs before neuronal loss. Therefore, synaptic deficit can be considered as starting event for neurodegeneration in α -synucleinopathies (Overk and Masliah, 2014; Bridi and Hirh, 2018). The relevance of synaptic damage in the pathogenesis of α -synucleinopathies is supported by a large amount of data, including the presence of α -synOs in axonal terminals which precedes the formation of LBs (Marui *et al.*, 2002), and the relationship between synaptic changes and α -synOs in pre-synaptic terminals, that ultimately lead to memory deficit in DLB (Kramer and Schulz-Schaeffer, 2007; Overk and Masliah, 2014; Bridi and Hirth, 2018). Altogether, these considerations allow the definition of α -synucleinopathies as synaptopathies, and ask for the elucidation of the mechanisms mediating α -synO detrimental effects at the synaptic level. In this regard, several mechanisms have been proposed which may co-exist and act in a synergistic manner. Through the binding of proteins involved in microtubule assembly and dynamics (tubulin, kinesin and MAP2), α -synOs promote an aberrant neurite network morphology and affect anterograde transport (Prots *et al.*, 2013). Synaptic vesicles derived from the Golgi apparatus get translocated to the axonal terminals by anterograde-mediated microtubules transport. Hence, alterations in the microtubule assembly and dynamics could explain the synaptic changes reported by Scott and colleagues in a mouse model over-expressing human α -syn (Scott *et al.*, 2010). Indeed, they found that high levels of α -synOs in the synaptic terminals were closely associated with reduction in synaptic vesicles density and synaptic proteins (Scott *et al.*, 2010).

In addition to microtubule alterations, α -synOs inhibit the synaptic docking and decrease neurotransmitter release. α -synOs interact with the vesicular protein sinaptobrevin-2, and as

a consequence they make the protein unavailable for the interaction with the t-SNARE complex, leading to inhibition of neurotransmitter release (Choi *et al.*, 2013).

Current knowledge suggests that multiple mechanisms at multiple cellular levels may be at play in the generation of α -synO-induced damages, supporting the hypothesis that α -synucleinopathies are multifactorial disorders even from a cellular viewpoint (Overk and Masliah, 2014; Bridi and Hirth, 2018).

2.6 Evidence for α -synOs in extracellular fluids and its implications

The cytosolic localization of α -syn and of its proteinaceous species suggests that cell-autonomous mechanisms could be involved in mediating α -synO detrimental activities. However, compelling evidence confirms the hypothesis that oligomers act also in a non-cell-autonomous manner. In fact, full-length α -syn could be detected in human extracellular fluids such as cerebrospinal fluid (CSF), interstitial brain fluids and blood (Borghi *et al.*, 2000; El-Agnaf *et al.*, 2003; Emmanouilidou *et al.*, 2011). Intriguingly, the release of α -syn occurs in PD patients as well as in healthy controls, therefore suggesting that α -syn secretion is a normal physiological process. Of note, the CSF α -syn pool is predominantly derived from brain neurons rather than peripheral blood (Mollenhauer *et al.*, 2012). The deep investigation of α -syn in extracellular fluids has also revealed that in CSF α -syn was detectable in its oligomeric state (Park *et al.*, 2011; Hansson *et al.*, 2014), further supporting the potential role of extracellular α -synOs in the pathogenesis of PD and PD-related disorders.

The detection of both monomeric and oligomeric α -syn in extracellular fluids of PD, DLB and healthy control patients is consistent with the evidence that neuronal cells release monomeric and oligomeric α -syn into the cell medium (El-Agnaf *et al.*, 2003, Lee *et al.*, 2005; Kim *et al.*, 2013), and with the finding that stress conditions increase the release

amount of α -synOs (Jang *et al.*, 2010). Several mechanisms have been put forward while attempting to describe the processes underlying α -syn release by neurons. Such mechanisms include the release of α -syn by dying neurons, unconventional exocytose type independent of the classical ER/Golgi pathway such as exosomes release, and autophagosome-mediated exocytosis (Danzer *et al.*, 2012; Gallegos *et al.*, 2015).

Based on α -syn behaviour and extracellular localization, two possibilities have been envisaged: I) α -synucleionopathies, as well as other protein misfolding related neurodegenerative disorders, may be a prion-like disease (Marques and Outeiro, 2012), and II) the potential of α -syn monomers and α -synOs as biological markers for the diagnosis at early stage and for the assessment of benefits deriving from new therapeutic approaches (Lee *et al.*, 2014).

Although the second point was received with excitement by the scientific community, at the state of the art, no reliable detection approaches have been developed to distinguish healthy control from individual PD or PD-related patients. That is because plasma and CSF levels of α -syn are comparable between healthy control and PD patients. Furthermore, levels of α -synOs are generally higher in PD patients but at the same time they may also overlap with healthy individuals, and therefore not useful as diagnostic tools to discriminate individual healthy controls to patients (Lee *et al.*, 2014). In contrast, Majbour and colleagues reported a novel antibody with a high sensitivity and specificity for α -synOs which allows the discrimination of PD from healthy individuals (Majbour *et al.*, 2016). On the other hand, the detection of α -synOs in CSF may be useful to distinguish patients with PDD or DLB by AD patients (Hansson *et al.*, 2014). Controversies often motivate research and provide new stimuli to push our knowledge forward. Validation of reliable biomarkers for early diagnosis and for monitoring the disease progression is a hot and urgent topic in the field of neurodegenerative disorders, thus requiring further investigation and innovation.

The findings reported here show that α -syn and α -synOs are normally secreted by neurons. Moreover, stress conditions increase the release of α -synOs, thus highlighting the potential role of extracellular oligomers in the pathogenesis of α -synucleinopathies and their potential significance as biological markers.

2.6.1 The non-cell-autonomous action of α -synOs

To explore the mechanisms involved in mediating α -synO detrimental activities, investigators mainly focused on the cell-autonomous processes at first. Nevertheless, the finding that α -synOs were detectable in extracellular fluids and the direct observation of released α -synOs by neurons suggested non-cell-autonomous mechanisms being at play in concert with the cell-autonomous ones.

Among cell types involved in non-cell-autonomous mechanisms, astrocytes and microglial cells have received increasing attention over the years. Such an interest raised from a number of observations, which include: the identification of reactive astrocytes and microglial cells in the brain of patients affected by different α -synucleinopathies (MacKenzie, 2000; McGeer and McGeer, 2008; Fellner *et al.*, 2011; Fellner and Stefanova 2013; Dickson, 2018); the detection of α -syn inclusions within glial cell bodies (Fellner *et al.*, 2011) and; the activation of both astroglial and microglial cells upon exposure to α -syn released by neurons (Alvarez-Erviti *et al.*, 2011; Fellner *et al.*, 2013; Kim *et al.*, 2013). Therefore, α -synOs may become neurotoxic *per se* or, as shown in figure 15, through an indirect route that involves activation of astroglial and microglial cells (Marques and Outeiro, 2012; Lim *et al.*, 2018).

Astrocytes and microglia are the main components of the innate immunity in the central nervous system (CNS). In response to various stimuli (*e.g.* lesions, infections, chronic stress...) they become activated and able to release several pro-inflammatory factors which may trigger neuronal dysfunction (Fellner *et al.*, 2011; Morris *et al.*, 2013; Blank and Prinz,

2013). Of note, activated glial cells contribute both to the onset and to the progression of the pathology. Through the production of pro-inflammatory signals and ROS, activated glial cells might lead to a detrimental microenvironment which in turn promotes the detrimental activities of oligomers (Lim *et al.*, 2018).

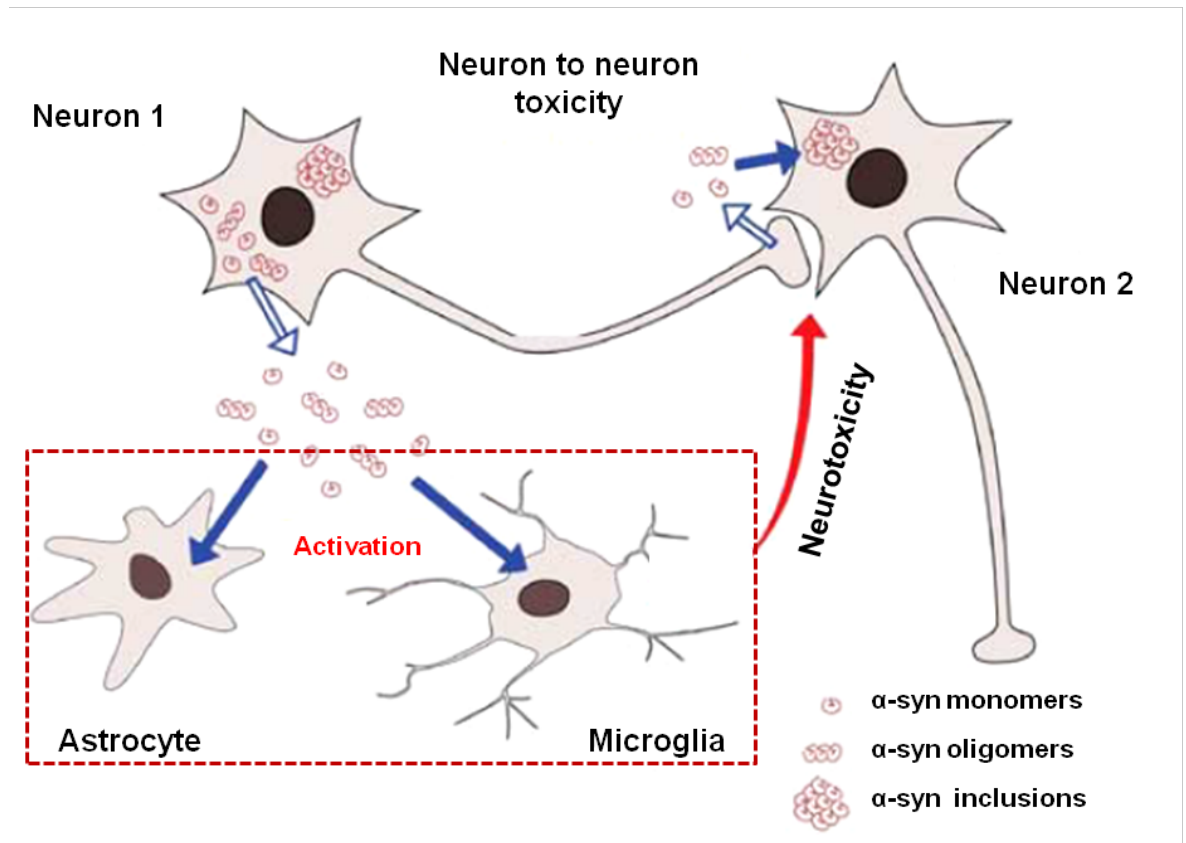


Figure 15. Schematic representation of the non-cell-autonomous action of α -synOs. Oligomers of α -syn can act through non direct mechanisms. They may be transferred between neurons or secreted by them. Extracellular α -synOs activate both microglial and astroglial cells, which in turn could lead to neuronal damage (modified from Marques and Outeiro, 2012).

Consistently, Kim and colleagues reported that the α -synO-conditioned medium derived from microglial cells leads to neuronal death *in vitro* (Kim *et al.*, 2016). More recently, di Domenico and co-workers demonstrated the pivotal role of astrocytes in mediating dopaminergic neuronal death. They indeed reported that induced pluripotent stem cells (iPSCs) -derived dopaminergic neurons show a reduction in the length of their neurites and an increase in cell death when co-cultured with iPSCs-derived astrocytes from PD patients

(di Domenico *et al.*, 2019). The relevance of glial cells in α -synucleinopathies is further supported by *in vivo* experiments showing that LPS-induced neuroinflammation in PD mouse models triggers dopaminergic neuronal loss in SN in association with increased neuroinflammation and oxidative stress (Gao *et al.*, 2008; Gao *et al.*, 2011). In addition, blocking microglial cell activation by drugs, such as minocycline, leads to a decrease in neuronal death (Lim *et al.*, 2016).

Collectively these findings support the idea that glia, in addition to neurons, may contribute with non-cell-autonomous mechanisms to the aetiology of α -synucleinopathies.

The implications of neuroinflammation for α -synucleinopathies as well as the role of astroglial and microglial cells in the regulation of neuronal functions will be further discussed in chapter III.

2.7 Alpha-synuclein oligomers and cognitive impairment

As previously described, progressive cognitive decline is a clinical feature of DLB and PDD and is an important element in consensus guidelines for the clinical diagnosis of both diseases (McKeith *et al.*, 2004). Changes in synaptic organisation have been described in DLB and PDD patients and have been linked with cognitive impairment (Kramer *et al.*, 2007; Schulz-Schaeffer, 2010).

Mounting evidence suggests a pivotal role for α -syn in the cognitive impairment. As mentioned above, over-expression of both WT and mutant forms of human α -syn is sufficient to induce memory deficits (Zhou *et al.*, 2008; Lim *et al.*, 2011; Larson *et al.*, 2012). Notably, Lim and co-workers demonstrated that suppression of α -syn rescued memory dysfunctions (Lim *et al.*, 2011).

The critical role of α -synOs in mediating memory damage has been demonstrated by different groups. Indeed, while LTP is significantly impaired in the hippocampus upon

exposure to α -synOs, monomers and fibrils are ineffective (Martin *et al.*, 2012; Diogenes *et al.*, 2012). Two different mechanisms have been proposed to explain such a detrimental effect: I) an increase in the calcineurin (CaN) activity, a phosphatase with a negative modulatory function on synaptic plasticity; II) an increase in basal synaptic transmission through an alteration in both AMPA and NMDA receptors permeability (Martin *et al.*, 2012; Diogenes *et al.*, 2012). In this regard, it was reported that the application of α -synOs in hippocampal cell cultures enhanced both pre- and post-synaptic AMPA receptor transmission (Hulls *et al.*, 2011), which might in turn lead to a calcium dyshomeostasis and cell death or synaptic loss as described in patients with DLB (Campbell *et al.*, 2000; Danzer *et al.*, 2007; Hulls *et al.*, 2011).

These data, obtained through electrophysiological approaches on brain slices, have been further reinforced by behavioural *in vivo* experiments. In fact, α -synOs intracerebroventricularly (ICV) injected in mice caused cognitive dysfunction as demonstrated using the fear conditioning memory task, whereas monomers and fibrils were again inactive (Martin *et al.*, 2012).

More recently, Ferreira *et al.* showed that the detrimental activity of α -synOs on LTP requires the association with the cellular prion protein (PrP^C) (Ferreira *et al.*, 2017). In particular, they found that PrP^C residues 93-109 are crucial to mediate LTP inhibition upon exposure to α -synOs, while α -synOs seem to require PrP^C to activate the Fyn kinase, which in turn leads to NMDAR2B phosphorylation giving rise to LTP inhibition. Moreover, Ferreira and colleagues, through immunoprecipitation approaches, demonstrated that α -syn and PrP^C can interact both in cell cultures treated with α -synOs and in a PD Tg mouse model overexpressing the human α -syn (Ferreira *et al.*, 2017).

Electrophysiological properties of neurons and of neuronal circuitries are tightly associated with intrinsic neuronal properties such as the expression of ion channels, neurotransmitter

release and receptor modulation. Deregulation of such a complex machinery may result in the alteration of neuronal plasticity and eventually in cognitive dysfunctions. While collectively these data support a key role for α -synOs in mediating cognitive impairment, a clear and univocal mechanism is still missing, and further investigation is required to acquire new insights on possible processes which may occur. On this subject, emerging data highlight the role of neuroinflammation as a potential culprit leading to neuronal dysfunction and impairment of cognitive functions. Pro- and anti-inflammatory cytokines, as well as LPS, have been reported to impair memory performance in different animal models (Donzis and Tronson, 2014; Richwine et al., 2009; Balducci *et al.*, 2018). The role of neuroinflammation in mediating memory damage is strongly supported by existing evidence in animal models of AD. In an acute mouse model of AD, amyloid- β oligomers (A β Os) have been reported to impair memory performance through activation of glial cells and increase of pro-inflammatory cytokines expression (Balducci *et al.*, 2017). Additionally, the block of neuroinflammation resulted in the rescue of memory functions both in an acute and in a Tg mouse model of AD (Balducci *et al.*, 2017; Balducci *et al.*, 2018). Intriguingly, α -synOs may exert detrimental activities through indirect non-cell-autonomous mechanisms which include glial cell activation. As mentioned above, activated glial cells produce several signals which can subsequently induce neuronal damage and impair cognitive functions. Thus, neuroinflammation might represent a process underlying cognitive decline in the context of α -synucleinopathies, and an exciting research direction to explain α -synO pathological mechanisms.

Introduction

Chapter III

3.1 Evidence for the role of inflammation in α -synucleinopathies

Initially considered as a mere secondary event and a consequence of the neurodegenerative process, neuroinflammation has acquired increasing interest in the context of α -synucleinopathies. This is partly due to the considerably big amount of data obtained from post-mortem analyses, *in vivo* brain imaging, assessment of pro-inflammatory mediators in human fluids, genetic/epidemiological studies and experimental animal models (Hirsch and Hunot, 2009; Dzamko *et al.*, 2015; Surendranathan *et al.*, 2015; Lim *et al.*, 2016).

A broad spectrum of stimuli is capable of eliciting an immediate and short-lived activation of the innate immune system within the CNS. Astrocytes and microglia act as brain's sentinels, and through the release of inflammatory mediators like cytokines and chemokines, they foster the migration of innate and adaptive immune cells such as monocytes/macrophages, neutrophils and lymphocytes (Walsh *et al.*, 2014). Although this response is normally self-limiting once the insult is resolved, persistence of inflammatory factors might result in pathological consequences and neurodegeneration (Glass *et al.*, 2010). The initiation of the inflammatory response generally occurs after the recognition by pattern recognition receptors (PRRs) of both exogenous (pathogen associated molecular patterns, PAMPs) and endogenous stimuli (damage associated molecular patterns, DAMPs). The binding of PRRs and DAMPs, such as misfolded and aggregated proteins including α -syn, leads to a signal transduction, which in turn triggers the activation of glial cells and the neuroinflammatory response (Heneka *et al.*, 2014). Of note, gliosis is a common feature of α -synucleinopathies (Fellner *et al.*, 2011). Microglial cell reaction is commonly observed in post-mortem PD brains (McGeer *et al.*, 1988; Chao *et al.*, 2014) and its persistence is linked to damaged dopaminergic neurons (Chao *et al.*, 2014). Moreover, in DLB cases increased microglial activation positively correlates with the load of LBs (Togo *et al.*, 2001). While the activation of the microglial pool in PD/DLB brains is widely accepted, contradictory results by different groups were reported for astrocytes which were found either in an active

or an inactive state (Tong *et al.*, 2015). In addition to astrogliosis and microgliosis, higher levels of pro-inflammatory mediators have been found in both the striatum and the SN of post-mortem PD and DLB brains (Togo *et al.*, 2001; Hirsch and Hunot, 2009).

Despite detecting the presence of an immune response within the brain, the neuropathological assessment of inflammation in post-mortem samples does not provide evidence enough to directly link neuroinflammation with the pathogenesis of α -synucleinopathies. Nevertheless, *in vivo* studies have clearly demonstrated that a neuroinflammatory process takes place during the progression of the disorders. PET scans with a ligand for microglia revealed the presence of activated microglial cells in PD, PDD and DLB patients (Hirsch and Hunot, 2009; Surendranathan *et al.*, 2015). Intriguingly, cortical microglial activation in PDD and DLB patients is characterised by a wider spreading compared to PD, and microglial activation negatively correlates with cognitive functions (Surendranathan *et al.*, 2015). Thus, a potential role for microglia in the pathogenesis of cognitive dysfunctions in both PDD and DLB has been speculated by researchers.

Analysis of pro-inflammatory mediators in central and peripheral human biological fluids provides further proof supporting a role of inflammation in the pathogenesis of α -synucleinopathies. Elevated levels of pro-inflammatory cytokines such as IL-1 β , IL-6 and TNF- α were identified in PD patients compared to age-matched healthy controls (Mogi *et al.*, 1994; Blum-Degen *et al.*, 1995; Mueller *et al.*, 1998; Clough *et al.*, 2013). Notably, levels of IL-6 are inversely correlated with the severity of the pathology in PD patients (Mueller *et al.*, 1998), and they are higher in PD cases with cognitive decline than in PD patients with no evidence of cognitive damage. Moreover, patients with cognitive dysfunction show an inverse correlation between IL-6 levels and the severity of the cognitive function impairment (Yu *et al.*, 2014). Since pro-inflammatory mediators appear increased and are detectable in biological fluids, they have been extensively investigated as potential biomarkers and diagnostic/prognostic tools. However, controversial results were published

(Brodacki *et al.*, 2008; Yu *et al.*, 2014; Gupta *et al.*, 2016; Hall *et al.*, 2018). These disparities may depend on several aspects such as the severity of the pathology, difference in the life-time spent with the pathology, as well as the presence of co-morbidities (Eidson *et al.*, 2017). In spite of inconsistent results in the alteration of some pro-inflammatory cytokines in biological fluids, the role of the inflammatory response in α -synucleinopathies has also been suggested by genetic and epidemiological studies. As summarised in table 4, genes involved in PD also play a role on glial cells (Joe *et al.*, 2018).



	Functions	PD genes that regulate glial functions
Astrocyte 	Glucose metabolism	PINK1, DJ-1
	Mitochondrial function	PINK1
	Intracellular ROS	parkin., DJ-1
	Growth factor production	DJ-1
	Astrogenesis	PINK1
	Astrogliosis	DJ-1
	Proliferation	PINK1, parkin
	Endocytosis	DJ-1
	Inflammation	DJ-1
Microglia 	Inflammation	DJ-1, PINK1, LRRK2, , α -syn
	Surveillance	LRRK2
	Phagocytosis	DJ-1, α -syn, LRRK2

Table 4. PD related genes and their function in astrocytes and microglial cells (Joe *et al.*, 2018).

Besides, several polymorphisms in inflammatory associated genes increase the susceptibility to develop PD (Hirsch and Hunot, 2009).

As described in chapter I, epidemiological studies have pointed out an inverse correlation between the use of NSAIDs, particularly Ibuprofen, and the risk of developing PD (Ascherio and Schwarzschild, 2016). On the other hand, systemic pathologies characterised by the induction of a neuroinflammation were found to positively correlate with an increasing risk of PD (McKenzie *et al.*, 2017).

Compelling evidence of neuroinflammation in PD also comes from studies in different animal models. Chronic overexpression of the cytokine IL-1 β in the SN of adult rat results in nigral dopaminergic degeneration and motor deficits, as well as glial activation (Ferrari *et al.*, 2006). The MPTP model of PD was shown to cause microgliosis and to increase the levels of inflammatory mediators in murine SN (Czlonkowska *et al.*, 1996; Mandel *et al.*, 2000). The Gram-negative bacterial endotoxin component LPS is a potent activator of inflammatory cells, and it has enabled researchers to gather information on the role of various inflammatory mediators in PD pathogenesis. As a matter of fact, the systemic administration of LPS has been reported to trigger a long-lasting increase in the pro-inflammatory cytokine TNF- α in the brain, an increase in microglial cell activation in hippocampus and SN, as well as a progressive dopaminergic neuronal loss in SN (Qin *et al.*, 2007). Interestingly, administration of LPS in mice overexpressing α -syn results in the enhancement of the dopaminergic neurodegeneration (Gao *et al.*, 2011). In addition, Tanaka and colleagues (Tanaka *et al.*, 2013) demonstrated that the sub-chronic intranigral administration of LPS in WT but not in IL-1 β null mice leads to microglial activation, increased pro-inflammatory cytokines production, death of dopaminergic neurons and motor behaviour impairment (Tanaka *et al.*, 2013). Of note, the same authors observed that microglial activation and motor deficits occur earlier than neuronal loss, thus describing neuroinflammation as an early phenomenon which triggers neuronal dysfunction and ultimately neuronal death (Tanaka *et al.*, 2013).

More findings confirming the involvement of neuroinflammation in PD have been provided exploiting Tg mouse models. In mice overexpressing WT human α -syn, microglial activation and high levels of the pro-inflammatory cytokine TNF- α in both striatum and SN were reported, as well as microgliosis and astrogliosis in the hippocampus (Gelders *et al.*, 2018; Kim *et al.*, 2018). Microglial cells seem to have a key role in dopaminergic neurodegeneration. In fact, onset of microglial activation appears before the dopaminergic

loss, and inhibition of microglial cell activation through minocycline administration results in a significant decrease of neuronal death (Wu *et al.*, 2002; Hirsch and Hunot, 2009). However, despite promising outcomes of minocycline in pre-clinical studies, a clinical trial with minocycline has proven unsuccessful (NINDS NET-PD Investigators, 2008). Therefore, further investigations of neuroinflammation in PD and PD-related disorders are mandatory to acquire full knowledge of the inflammatory mechanisms underlying these disorders.

3.2 Astrocytes and microglial cells are key players in the healthy brain

Together with oligodendrocytes, which isolate axons and allow the fast and efficient propagation of the action potential, astrocytes and microglial cells are the main cellular components of the CNS and have essential functions in maintaining brain homeostasis and properties (Bacci *et al.*, 1999; Hertz and Chen, 2016).

Despite their different embryogenic origin, both astrocytes and microglial cells are immune competent cells in the brain, and they are crucial players in the fine regulation of neuronal activity and synaptic plasticity (Blank and Prinz, 2013; Hertz and Chen, 2016). Synapses are specialised structures involved in the processing and transmission of the information between neurons and, furthermore, they are involved in learning and memory. Thus, a strict survey and tuning of synaptic functions is a milestone for superior functions.

In healthy brains, astrocytes as well as microglial cells are juxtaposed and directly contact synapses at both pre- and post-synaptic levels (Figure 16).

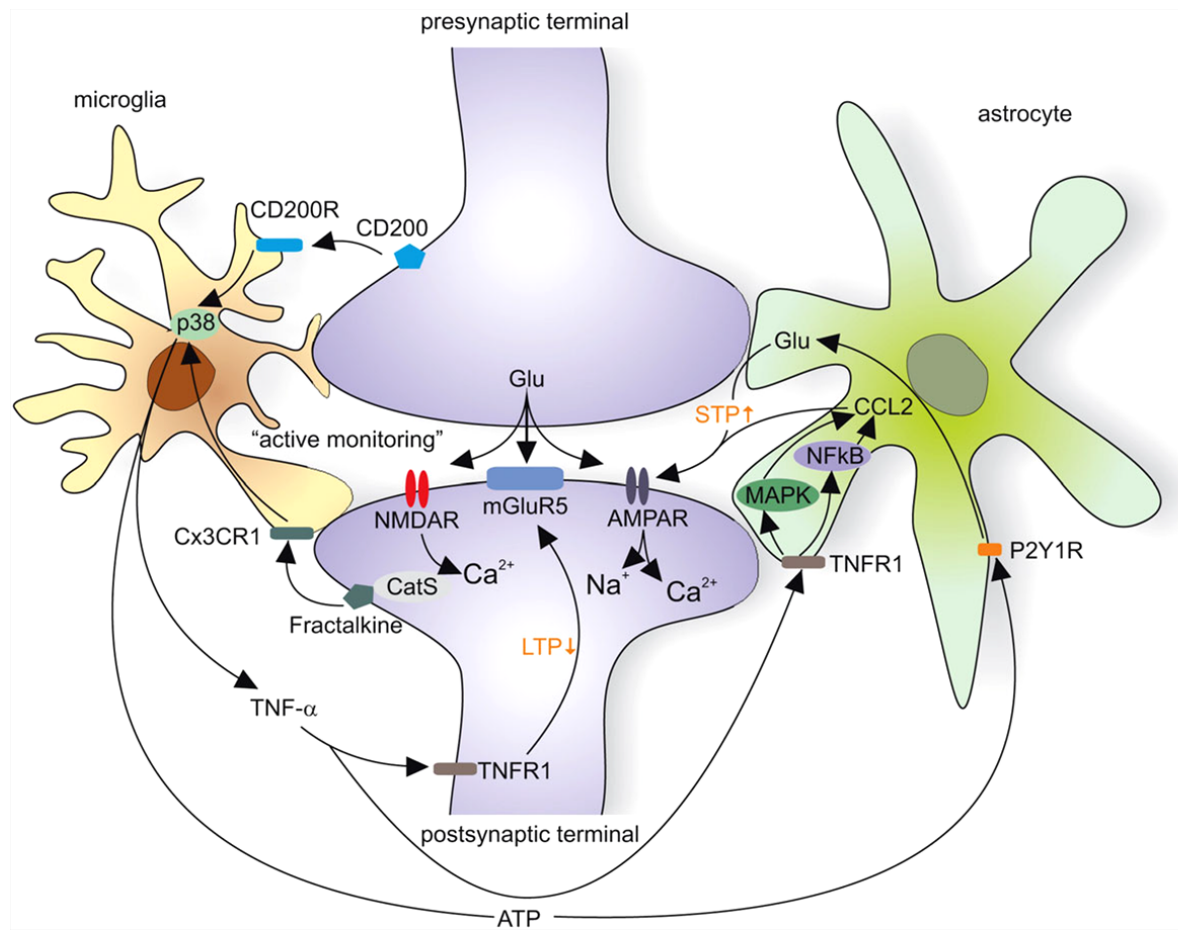


Figure 16. Schematic representation of the tight interconnection between neurons, astrocytes and microglial cells at synaptic level. Astrocytes and microglial cells finely survey synaptic activity and tune it as well as synaptic plasticity. Of note both microglial and astrocyte activities at synaptic level are dependent on neuronal activity and is regulated by direct and indirect interconnections among all three cell types (Blank and Prinz, 2013).

A single astrocyte may contact up to two million synapses in human and, such a spatial organisation allows the constant monitoring of synaptic activity, which in turn is essential for tuning synaptic functions and plasticity (Blank and Prinz, 2013; Hertz and Chen, 2016). Astrocytes are essential for providing a metabolic support to neurons. Namely, astrocytes uptake glucose from the blood and convert it into lactate through glycolysis. After release into the extracellular space, lactate is then utilised in the oxidative metabolism by neurons (Bacci *et al.*, 1999; Joe *et al.*, 2018). Astrocytes have also an important function in the removal of glutamate from the synaptic cleft. In presence of a sustained neuronal activity, a

high release of the excitatory neurotransmitter glutamate takes place. Glutamate is removed from the synaptic cleft by astrocytes through a transporter linked with a Na^+ inward current. The resulting increase in the intracellular Na^+ concentration leads to the activation of the Na^+/K^+ ATPase which in turn stimulates glycolysis and the production of lactate. Thus, this fine mechanism clearly demonstrates that astrocytes provide lactate to neurons in a synaptic activity-dependent manner (Bacci *et al.*, 1999). In addition, astrocytes are involved in ion homeostasis, in regulating brain water volume and in modulating oxidative stress through the production of glutathione. Moreover, astrocytes provide glutamine to neurons which is converted by neurons in glutamate and produces diverse neurotrophic factors essential for neuronal health (Joe *et al.*, 2018).

Aside of astrocytes, also microglial cells have vital functions in healthy brain. In its resting state, microglial cells interplay a large number of connections with both neurons and astrocytes, and they play a role in synaptic remodelling as well as in providing neurotrophic factors to neurons. When in a resting state, microglial cells have a ramified morphology with a high number of branching processes and a small body. The extremely dynamic and mobile thin processes of microglial cells constantly survey the microenvironment and the synaptic activity. Indeed, microglial cells have receptors for several neuronal transmitters and through different signals, including inflammatory mediators, may regulate the neuronal activity (Morris *et al.*, 2013). Of note, microglial cells in healthy conditions produce physiological levels of pro- and anti-inflammatory cytokines such as $\text{IL-1}\beta$, IL-10 and $\text{TNF-}\alpha$, which are involved in synaptic plasticity, learning and memory (Blank and Prinz, 2013; Donzis and Tronson, 2014). Therefore, alteration of number, morphology and activation state of microglial cells, which generally occur during chronic and not regulated inflammation, may lead to synaptic and neuronal damage that ultimately may result in neurodegeneration (Blank and Printz. 2013; Morris *et al.*, 2013).

Microglial activity at synaptic level is regulated by several mediators. Among these, neuronal fractalkine and CD200 have an inhibitory effect on microglial activation. The fine regulation of microglial cells is essential for the neuronal and synaptic functions and it involves neurons, astrocytes and microglial cells themselves. Thus, impairment of this fine and complex mechanism may trigger impaired synaptic plasticity, LTP and in turn memory damage (Blank and Prinz, 2013; Morris *et al.*, 2013; Yang *et al.*, 2007).

As described in Chapter II, synaptic plasticity is an essential process for learning and memory. During learning and memory formation many events including creation of new synapses, elimination of “unwanted”, not well-working or damaged synapses and synaptic remodelling take place. Of note, microglial cells and astrocytes maintain phagocytic properties during adulthood, thus allowing remodelling, refinement and clearance of synapses (Jung and Chung, 2018).

3.3 General characteristic of microglial cells

Neuroinflammation triggered by microglial cells is a key player in PD and DLB pathogenesis. In fact, microgliosis positively correlates with dopaminergic neurodegeneration in SN (Ouchi *et al.*, 2005).

Microglia derive from primitive yolk sac myeloid progenitors which seed in the developing brain parenchyma where they persist. Of note, microglial cells are exclusively present in the CNS and are not found in other organs or tissues (Subramaniam and Federoff, 2017). Microglial cells represent 10-15% of cells in the brain, and while they are evenly distributed, their density and morphology vary across brain regions (Tremblay *et al.*, 2011; Subramaniam and Federoff, 2017). In the healthy brain, microglial morphology and distribution are dependent on the local cytoarchitecture. While in high dense cell regions microglia occur with a low rate, microglial cells are abundant in low dense cell fields. Thus, indicating a negative correlation between local cell density and microglial distribution into

the brain (Tremblay *et al.*, 2011). As mentioned above, local cytoarchitecture also influences the morphological aspect of microglia. In white matter, microglia show an elongated soma with processes oriented along fibres. In contrast, in grey matter microglial cells have an elaborated shape with many radially oriented processes (Tremblay *et al.*, 2011). Microglial cells, similarly to peripheral macrophages, survey the microenvironment against pathogens and damage signals. This function is closely related to the ability of microglial cells, at least in their resting state, to perpetually change their morphology by extending and retracting their own highly motile processes (Tremblay *et al.*, 2011; Morris *et al.*, 2013; Subramaniam and Federoff, 2017). Despite the high motility of their processes, microglia have an individual territory and direct contacts between microglial processes are avoided (Tremblay *et al.*, 2011). In response to both exogenous and endogenous stimuli, resting microglial cells are activated and undergo a changing process. This includes alterations in both cellular morphology - from a ramified to an ameboid shape with an enlargement of the soma - and in gene/protein expression profile which in turn underlies the brain immune response. Since fine tuning of the immune response in both its initiation and resolution is crucial for the clearance of harmful elements as well as for brain homeostasis, an uncontrolled balance of microglial activation may result in brain damage and neurodegenerative disorders including PD (Cherry *et al.*, 2014; Tang and Le, 2016).

3.3.1 The dual role of microglial activation

Resting microglia can either be activated in a classical activation way leading to an M1 proinflammatory microglial phenotype, or via an alternative way, that results in an M2 anti-inflammatory microglial phenotype (Figure 17). In addition to the classical and alternative activation, microglial cells may also undergo an acquired deactivation process which is essential for the resolution of the immune response and results in the switching of the M1 microglia to the M2 microglial phenotype (Tang and Le, 2016).

M1 microglia are characterised by pro-inflammatory and pro-killing functions that serve as a rapid prime line defence. Several kinds of signals may lead resting microglia to acquire an M1 phenotype including IL-1 β , TNF α , interferon gamma (IFN γ) as well as α -synOs (Kim *et al.*, 2013; Subramaniam and Federoff, 2017). In this activated state, the production of pro-inflammatory mediators is significantly increased and several markers enabling the identification of M1 microglial cells are overexpressed (CD16/32, inducible nitric oxide synthase iNOS, IL-1 β , TNF α , IL-6...). In contrast to M1 microglia, M2 microglial cells are associated with the resolution of the neuroinflammation. In fact, in this activation state microglial cells express anti-inflammatory mediators and enzymes such as IL-4, IL-10, IL-13, transforming growth factor β (TGF β), arginase 1, YM1, receptors associated with the phagocytosis. All these signals, apart from being useful M2 microglial markers, are involved in dampening neuroinflammation, in resolving injury as well as in repairing tissues (Cherry *et al.*, 2014; Subramaniam and Federoff, 2017). Of note, anti-inflammatory cytokines have a double function. Namely, they counteract the production of pro-inflammatory mediators and, on the other hand, they promote the acquisition of an M2 phenotype (Cherry *et al.*, 2014; Tang and Le 2016). The fine and complex regulation of M1/M2 microglial phenotypes is crucial for brain homeostasis and health. A clear example of such a fine process is provided by the cross-talk between iNOS and arginase 1, that are expressed by M1 and M2 microglia, respectively. Both iNOS and arginase 1 are involved in the metabolism of arginine, which represents the unique substrate for both enzymes. The metabolism of arginine by iNOS leads to the production of citrulline and nitric oxide (NO). Of note, NO is toxic on neurons, and it was reported to be involved in dopaminergic neurodegeneration through different mechanisms (Zhang *et al.*, 2006b; Choi *et al.*, 2009). Unlike iNOS, arginase 1 converts arginine in hydroxyproline, proline and polyamine that contribute to tissue repair (Tang and Le, 2016). As arginine is the substrate of both iNOS and arginase 1, the latter counteracts NO production and may prevent neuronal damage.

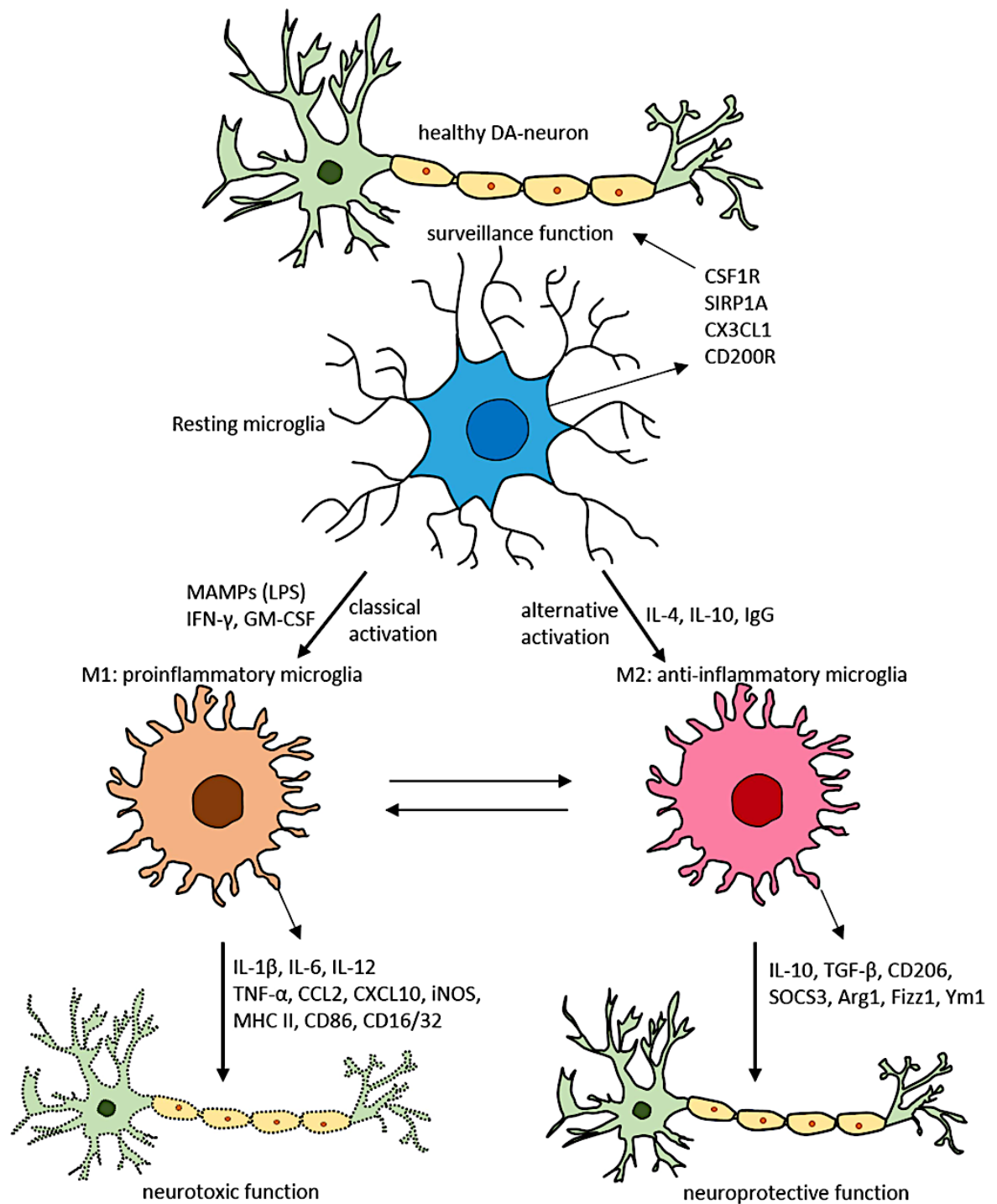


Figure 17. Schematic representation of the classical and alternative pattern of microglia. In response to diverse stimuli, microglia may acquire an M1 or an M2 phenotype which are associated with a different protein expression as well as a divergent effect on neurons (modified from Subramaniam and Federoff, 2017).

Based on the observation of microglial activation in PD and on the observation of possible different microglial phenotypes, researchers are attempting to further understand the

differential role of M1 and M2 microglia in PD. In this regard, it has been recently reported in an LPS-induced PD mouse model that the progressive nigrostriatal dopaminergic neurodegeneration is linked to an M1 microglial phenotype. In contrast, the cessation of the dopaminergic neurodegeneration strictly occurs in association with the switch of microglial cells to an anti-inflammatory M2 phenotype (Beier *et al.*, 2017). In addition to the different properties of activated microglia, an emerging concept in the field of microglial cells and neurodegenerative disorders is the “priming” of microglial cells. Microglia, indeed, may retain features acquired during previous immune challenges, thus becoming “primed” and respond to a subsequent stimulus in an exaggerated manner. In turn, such a strong reaction may ultimately result in an uncontrolled inflammatory response that may become chronic. Once the inflammatory response becomes chronic, it self-sustains through a vicious circle and leads to a harmful microenvironment being dangerous for neurons (Tremblay *et al.*, 2011; Lecours *et al.*, 2018).

3.4 Brain and peripheral inflammation: a strong connection

Neuroinflammation has a key role in the pathogenesis of neurodegenerative disorders including α -synucleinopathies. Of note, peripheral inflammation contributes and increases brain inflammation, thus suggesting a tight and complex cross-talk between the two immune systems (Kempuraj *et al.*, 2017). The direct link between systemic and brain inflammation raises from a lot of evidence comprising data from both human samples and experimental animal models. As previously described, elevated levels of several pro-inflammatory mediators have been detected in both blood and CSF of PD patients. In addition, the induction of a peripheral inflammation through a single intraperitoneal injection of LPS triggers a self-propelling chronic neuroinflammation which is accompanied by dopaminergic neurodegeneration. Remarkably, the LPS-induced neuroinflammation seems to be weakly linked to the LPS *per se*, but instead appears more closely related to the

expression of the pro-inflammatory mediator TNF- α , which remains elevated in the brain for a long time after LPS treatment (Qin *et al.*, 2007).

Speaking about the different peripheral immune cells, monocytes have acquired an increasing interest in PD pathogenesis. Monocytes are a subset of myeloid cells which represent the main component of the peripheral innate immune system (Wijeyekoon *et al.*, 2018; Mahad *et al.*, 2006). In response to specific signals these cells are recruited from the periphery into the brain where they contribute to the neuroinflammatory response (Harms *et al.*, 2018). One of the best known and well-established pathways involved in the brain monocyte recruitment is the CCL2/CCR2 pathway. Through CCR2, peripheral monocytes recognise the circulating chemokine CCL2, which in turn promotes the migration of monocytes across the blood-brain-barrier (Wijeyekoon *et al.*, 2018; Harms *et al.*, 2018). Once inside the brain, monocytes may differentiate into active macrophages, which contribute to neuroinflammation and therefore could be detrimental for brain integrity. Intriguingly, CCR2 expression is elevated in peripheral blood monocytes of PD patients compared to healthy controls (Funk *et al.*, 2013), and monocytes from the blood of PD patients show a higher production of CCL2 than those of age matched healthy controls (Reale *et al.*, 2009). As a consequence, such an alteration in the expression of both CCL2 and CCR2 may contribute to the self-propelling of the inflammation in both periphery and brain. A significant evidence of the role of monocytes in PD has been recently provided by Harms and colleagues. Exploiting a PD mouse model overexpressing human α -syn, they demonstrate a high recruitment of peripheral monocytes in the SNpc. Moreover, genetic deletion of CCR2 hampered the α -syn-induced monocyte recruitment, neuroinflammation and dopaminergic neurodegeneration (Harms *et al.*, 2018). These lines of evidence shed new light into peripheral inflammation in PD pathogenesis. However, a contribution of both peripheral and brain inflammation seems to be more plausible than the role of solely

peripheral monocytes in PD pathogenesis. In fact, data indicate that α -synOs, as previously described, directly interact with and activate microglia.

3.5 The Toll-like receptors

As mentioned above, the initiation of the inflammatory response generally occurs after the recognition of DAMPs or PAMPs by PRRs such as TLRs. TLRs are first line players involved in the innate immune response and are able to detect both exogenous and endogenous signals including misfolded and aggregated α -syn. TLRs are highly conserved throughout species ranging from nematodes to higher eukaryotes, and they are the best characterised group of innate immune receptors. To date, 10 and 13 functional TLRs have been discovered in humans and mice, respectively (Arroyo *et al.*, 2011). They are type I transmembrane proteins with a leucine-rich ectodomain recognising PAMPs/DAMPs, a transmembrane domain, and an intracellular Toll-interleukin 1 receptor (TIR) involved in downstream signalling (Arroyo *et al.*, 2011). The outcome of signalling is determined by the cell type expressing the receptors and through the selective use of specific sorting adaptor proteins. In addition, the response specificity is achieved through the subcellular compartmentalisation of the receptors themselves. For example, TLRs involved in the recognition of nucleic acids (TLR3, 7, 8 and 9) are normally found on the membrane of intracellular endo-lysosomes, while TLRs recognising extracellular PAMPs/DAMPs (TLR2, 4, 5, 6) are expressed on the cell surface facing the extracellular environment (Arroyo *et al.*, 2011). A large body of evidence indicates that TLR2 and TLR4 are the most frequently receptors involved in the pro-inflammatory immune response triggering neuroinflammation. Accordingly, microglia lacking TLR2 and TLR 4 fail to mount an immune response upon exposure to fibrillar A β ₁₋₄₂ (Jana *et al.*, 2008). Moreover, A β O-induced memory damage and neuroinflammation have both been reported to be mediated by the TLR4 (Balducci *et al.*, 2017). While TLR2 agonist is often represented by bacterial

lipoprotein, the classical PAMPs for TLR4 is the LPS. Upon binding to circulating lipid binding protein (LBP), LPS is engaged in a complex with membrane-bound CD14, that in turn combines with TLR4 to build up a functional LPS receptor complex. The downstream signalling involves an intricate network of adaptor molecules, protein kinases and phosphatases leading to the final activation of NF- κ B and AP1 transcription factors that promote the expression of pro-inflammatory genes (Rosadini *et al.*, 2017).

Microglia and astrocytes are the main cell types mediating innate immunity in the CNS. Studies in mice revealed that mRNAs of TLR 1-9 are expressed in the brain, whose expression can be up-regulated by infections and inflammation, thus amplifying the innate immune response (Trudler *et al.*, 2010; McKimmie *et al.*, 2005).

As previously described, microglia are first line responder cells to various kind of insults representing the 10% of cells in an adult CNS, and they are considered the brain's macrophages (Aguzzi *et al.*, 2013). These cells, expressing TLRs 1-9 along with CD14, respond to stimulation by producing cytokines that finally trigger phagocytosis of microorganisms and aggregated extracellular proteins (Arroyo *et al.*, 2011). Microglia harbouring various TLRs are present in different brain areas with some preference to regions close to the circulation as meninges and circumventricular organs (Arroyo *et al.*, 2011).

Aside from microglial cells, astrocytes have been found to express TLRs at lower levels in physiological conditions. In healthy human brains these receptors are barely present, but upon inflammation onset TLRs are expressed on the surface of astrocytes at levels even detectable by immunohistochemical approaches (Arroyo *et al.*, 2011).

Of note, TLRs are also present in neurons, further providing evidence that they have a function in maintaining the cerebral architecture and tissue homeostasis. Specifically, humans and rodents express TLR 2, 3 and 4 in their neurons, while mRNAs coding for TLRs 1-8 have been found in rat and mouse primary neuronal cultures (Tang *et al.*, 2007; Okun *et al.*, 2009). It has been established that TLRs activation in neurons might work in brain

development, and that in murine CNS TLR3 is involved in the regulation of the growth-collapse rate of neuronal cones (Arroyo *et al.*, 2011).

3.6 Altered expression of TLR2 and 4 in Parkinson's disease

Despite the contribution of TLRs in α -synucleinopathies is controversial and debated, evidence for TLR engagement in such pathologies raises from the observation of TLR altered expression in *in vitro* studies as well as in PD patients and animal models (Kouli *et al.*, 2019).

Treatment of primary microglial cells with a preparation of α -syn containing a mixture of aggregates, from monomeric to fibrils, leads to microglial activation and altered transcriptional profile of TLRs including increased expression of TLR2 and a down-regulation of TLR4 (Beraud *et al.*, 2011). An altered expression profile of TLRs has been also previously reported by Letiembre and co-workers (Letiembre *et al.*, 2009). In the brainstem of a Tg PD mouse model these authors found an increase in both transcriptional and protein levels of the TLR2, whereas the expression of the TLR4 was unaffected (Letiembre *et al.*, 2009). More recently in PD patients, increased levels of both TLR2 and 4 have been detected in blood peripheral immune cells. However, while the expression of TLR2 was significantly higher in monocytes compared to healthy matched controls, TLR4 expression in the same cells revealed solely a trend in its up-regulation (Drouin-Ouellet *et al.*, 2015). Despite the different expression profile of TLR2 and 4 in the blood, in the same study Drouin-Ouellet and colleagues found a comparable increase in protein levels of both TLRs in the caudate/putamen of post-mortem PD brains. Nevertheless, the same authors could not confirm their finding in a Tg mouse model over-expressing the WT human α -syn (Drouin-Ouellet *et al.*, 2015). On the other hand, a different TLR2 expression profile has been reported by Doorn and colleagues. They found that, post-mortem brain samples from patients with prodromal and diagnosed PD displayed stage-dependent expression of TLR2

in both the SN and in the hippocampus, with a higher TLR2 expression in prodromal PD compared to diagnosed and advanced PD (Doorn *et al.*, 2014). Therefore, these data imply that expression of TLR2 in early or advanced PD could be region- and time-specific.

Because of controversial results being reported on the involvement of TLR2 and 4 in PD, in the following paragraph I will provide a brief overview on these two receptors in the context of the pathology.

3.6.1 Experimental evidence for TLR2 and TLR4 involvement in α -syn detrimental effects

As described in chapter II, extracellular α -syn could exert its detrimental activities on neurons through non-cell-autonomous mechanisms which recruit microglial and astroglial cells. Activation of these cells by α -syn may occur through the signal transduction mediated by either TLR2 or TLR4. In two distinct papers, Kim and co-workers have demonstrated that α -synOs released by SHSY-5Y cells over-expressing human WT α -syn promote microglial cell activation and the expression of pro-inflammatory mediators in a TLR2-dependent manner (Kim *et al.*, 2013; Kim *et al.*, 2016). Indeed, genetic ablation of the receptor as well as its functional inhibition completely abrogated the microglial cytokine production upon α -synOs treatment (Kim *et al.*, 2013; Kim *et al.*, 2016). In contrast, as the depletion of TLR4 had no effects on the α -syn-induced gene expression of microglial pro-inflammatory cytokines, they concluded that α -synOs act through a TLR4-independent pathway (Kim *et al.*, 2013). In post-mortem PD brain, TLR2 is up-regulated in both microglia and neurons, even though is more expressed in neurons compared to microglia (Dzamko *et al.*, 2017). In addition, neuronal stimulation through TLR2 agonists leads to a significant increase in the expression of pro-inflammatory mediators by neurons themselves, thus depicting neurons as active players in the neuroinflammatory process in concert with glial cells (Dzamko *et al.*, 2017). Evidence for the involvement of TLR2 in α -syn detrimental

actions also stems from *in vivo* studies. Indeed, while TLR2^{+/+} mice overexpressing the WT human α -syn in the SN showed loss of dopaminergic neurons and active microglial cells, TLR2^{-/-} mice did not (Kim *et al.*, 2013). As described in the above section, TLR2 levels are increased in PD patients. These data and the evidence of TLR2-dependent activation of microglial cells by α -synOs allow researchers to speculate that TLR2 may play a pivotal role in promoting neurodegeneration in α -synucleinopathies. To follow up this hypothesis, it has been recently demonstrated in a Tg PD mouse model that TLR2 blockade through a functional inhibiting antibody triggers neuroprotective effect. Specifically, immunotherapy against TLR2 leads to the inhibition of both microgliosis and astrogliosis as well as the normalisation of IL-1 β , IL-6 and TNF- α levels (Kim *et al.*, 2018). Such an overall effect on the neuroinflammatory parameters was also accompanied by a significant reduction of cortical and hippocampal neuronal degeneration (Kim *et al.*, 2018).

The data here provided seem to suggest a prominent role for TLR2 in α -synucleinopathies, albeit TLR4-dependent activation of microglia and astrocytes upon α -syn treatment has also been reported. The exposure of both TLR4^{+/+} microglial cells and astrocytes to different moieties of WT α -syn (soluble full-length, oligomers and C-terminally truncated) triggers their activation and the release of pro-inflammatory mediators such as TNF- α and IL-6. Remarkably, ablation of the receptor results in the suppression of the pro-inflammatory response in both cell types (Fellner *et al.*, 2013). Consistently, the involvement of TLR4 in mediating the astrocyte α -syn-induced pro-inflammatory response has also been reported by Rannikko and collaborators (Rannikko *et al.*, 2015). In their study the authors described an α -syn dose-dependent increase in the transcriptional levels of different pro-inflammatory mediators such as IL-1 β , IL-6, TNF- α and COX-2 in TLR4^{+/+}, but not in TLR4^{0/0} astrocytes (Rannikko *et al.*, 2015). Thus, contradictory results leave the involvement of TLR2 and TLR4 in α -synucleinopathies still unclear and demand further efforts to clarify their contribution in mediating α -syn detrimental activity.

Aims of the study

Chapter IV

Mounting evidence depicts α -synOs as key players in inducing neuronal and synaptic dysfunction in α -synucleinopathies, and highlights the potential harmful effects of extracellular α -syn moieties. Therefore, the first aim of this PhD thesis was to develop an acute mouse model based on the ICV injection of α -syn monomers, oligomers and fibrils to specifically investigate the oligomeric hypothesis in the context of cognitive damage.

Since researchers have started focusing on the mechanisms involved in mediating the dangerous action of α -synOs, non-cell-autonomous mode of actions gained an increasing interest. Among these, neuroinflammation and protein-protein interaction are being widely explored and currently proposed as crucial events in the pathogenesis of PD and PD-related disorders. Specifically, with regard to protein-protein interaction, the PrP^C was recently shown to interact with α -synOs, and to mediate their effects at a functional level. Based on these data, further aims of this PhD thesis included:

- The elucidation of α -synOs ability to induce a neuroinflammatory response in our acute mouse model, and the characterisation of the role of α -synO-induced neuroinflammation in mediating the detrimental actions of α -synOs on memory.
- The *in vivo* investigation of the PrP^C- α -synO interaction to assess the role of PrP^C as an interactor and mediator of oligomeric α -syn at multiple functional levels (neuronal cytotoxicity, memory damage and gliosis).

As mentioned above, neuroinflammation has emerged as a key player in PD and PD-related disorders pathogenesis. Although neuroinflammation may represent a mediator for α -synOs, compelling evidence supports a role of neuroinflammation as a bridge linking environmental and genetic susceptibility co-fostering PD pathogenesis. These findings together with the lack of direct evidence demonstrating that inflammation influences α -synO harmful activities or the PD behavioural and neuropathological features, set the stage for the next aim of this PhD thesis. Specifically, we intended to verify whether an induced inflammatory state potentiates α -synOs effects in our acute mouse model, and whether it influences the PD

phenotype in the more complex context of the Tg PD mouse model carrying the A53T missense mutation.

In conclusion, the overall aims of this PhD thesis were to investigate the action of extracellular α -synOs at cognitive level, and to elucidate their mechanisms of action focusing on neuroinflammation as a mediator of α -synOs effects and as a predisposing factor influencing α -synO actions and pathology progression (Figure 18).

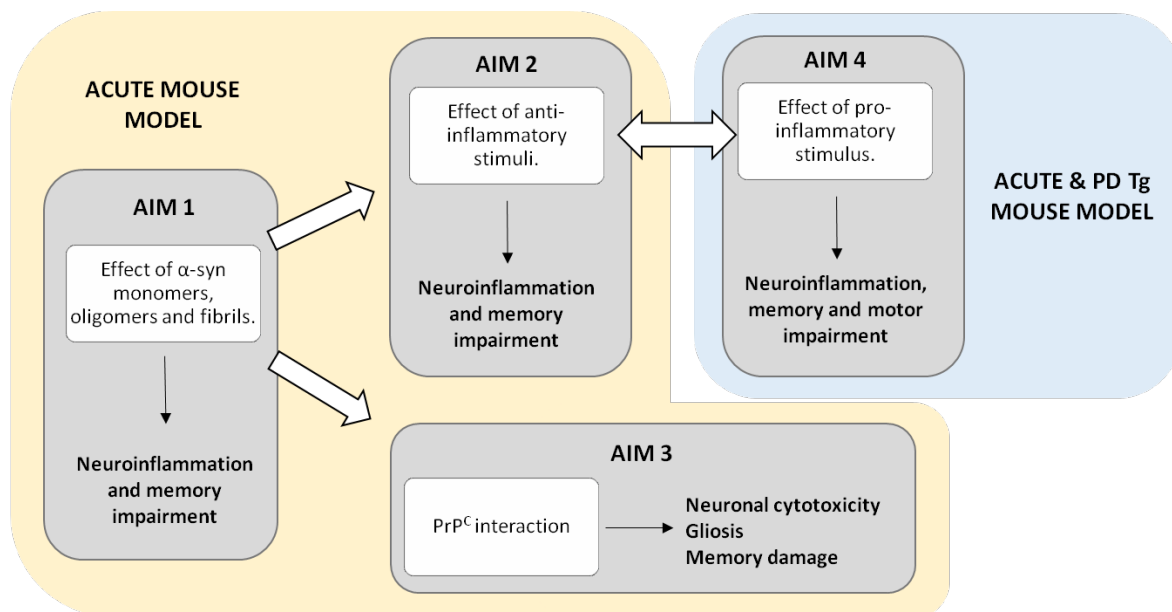


Figure 18. Overall aims of the PhD thesis

Materials and Methods

Chapter V

5.1 Mice

In this PhD thesis different mouse strains were exploited as specified in each result section. In particular mice used for our experiments included: eight-week-old C57BL/6N (Charles River, Italy) and TLR4 knock-out (TLR4^{0/0}) male mice (kindly provided by Dr Vezzani, Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy); six-/eight- months old PrP^C knock-out (*Prnp*^{0/0}) and *Prnp*^{+/+} male mice (Bueler *et al.*, 1993) maintained on a pure C57BL/6 background were obtained by the European Mouse Mutant Archive (strain EM01723), and eight-month-old hemizygous B6.Cg2310039L15Rik^{Tg(Prnp-SNCA*A53T)23Mkle/J} (herein referred as A53T mice) and their non-transgenic matched littermates (NTG) (The Jackson Laboratory, USA). Mice were housed individually in standard cages in a specific pathogen free conditions and in a controlled environmental condition (temperature: 21±1°C; relative humidity: 60% and 12 hours of light). All experimental procedures were conducted in conformity with institutional guidelines that are in compliance with national (D.L. n.26, G.U. 4 March 2014) and international guidelines and laws (EEC Council Directive 86/609, OJ L 358, 1, 12 December 1987, Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996), and were reviewed and approved by the intramural ethical committee.

5.2 ICV cannulation

Mice were anaesthetised with isoflurane inhalation and fixed on stereotaxic instrument (model 900, David Kopf instrument). A stainless steel guide cannula was implanted following the coordinates of the mouse cerebral atlas (anteroposteriority: +0.3, and laterality: ±1.0 from Bregma; dorsoventrality: -3.0 from Dura). To allow the recovery of animals from the surgery, animals entered in the experimental protocol 15 days after the surgical procedure.

5.3 Alpha-syn assembly preparations

α -synOs were obtained as previously described (La Vitola *et al.*, 2018; La Vitola *et al.*, 2019). Briefly, endotoxin free recombinant monomeric human α -syn (241 μ M) from *E. coli* cells (kindly provided by Prof. Pollegioni, Università degli Studi dell'Insubria, Varese, Italy; Caldinelli *et al.*, 2013) was incubated in 50 mM filtered phosphate buffer (PBS) pH 7.4 at 37°C without shaking for 48 hours. An incubation of 5 days in the same conditions used for obtaining α -synOs was applied to induce the fibrilization of recombinant monomeric human α -syn. At the end of the oligomerisation/fibrilization process, α -syn preparations were diluted at 1 and 0.5 μ M (as specified in each result section) in 50 mM filtered PBS (Vehicle, Veh) for the ICV injection. For *in vitro* studies, α -synOs were diluted at 1, 5 and 10 μ M in the neuronal maintaining medium.

Recombinant human α -syn was characterised by Prof. Pollegioni (Università degli Studi dell'Insubria, Varese, Italy) through size-exclusion chromatography on a Superdex 200 HR 10/30 column (GE Healthcare), SDS-PAGE and circular dichroism analyses. Endotoxins were fully eliminated by treatment with Triton X-114. α -synOs and fibrils were characterised by atomic force microscopy (AFM) analyses. In addition, α -synOs were characterised by western blotting.

5.4 Atomic force microscopy

AFM characterisation of our preparations were performed by Dr. Laura Colombo (Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano, Italy). A Nanoscope® Multimode V AFM equipped with a 7415 EV scanner (Veeco, NY, USA) was utilised to investigate the surface morphology of α -syn samples. Protein solutions containing oligomers or fibrils were diluted to 10 μ M with 50 mM PBS or 10 mM HCl, respectively. Once diluted, 50 μ L of each solution were adsorbed onto freshly cleaved mica (three minutes for the solution containing α -synOs, and five minutes for the solution containing fibrils). Then samples were washed

with 10 ml of double distilled H₂O and dried under gentle flow nitrogen. AFM measurements were performed using the tapping mode with a triangular antimony doped silicon cantilever (type-RTESP, Bruker). The force constant of the cantilever was 20-80 N/m with a resonance frequency of 313 - 370 kHz. The scan rate was 0.7 to 1 Hz, and the image size was 512 x 512 pixels. Images were obtained from three different locations on each specimen to ensure the homogeneity of samples. Analyses of AFM images were performed using Scanning Probe Image Processor (SPIP-version-5.1.6) data analysis package. Through the same approach we have also performed analyses of α -synO preparations with a final concentration of 1, 5 and 10 μ M.

5.5 Western blotting of α -synOs

Double gradient gel (7-12% SDS-PAGE) was used for the analysis of synthetic α -synOs. 3.75 μ g of α -syn were run in duplicate, transferred on nitrocellulose membranes, and probed using the mouse anti- α -syn antibody (1:1000, 4°C ON; BD Transduction Laboratories in BSA 3%). Then the membranes were incubated with the anti-mouse HRP secondary antibody (1:5000; 1 hour at room temperature, RT; Abcam). Immunoreactivity was visualised with Lumnia Forte Western HRP substrate (Millipore, Billerica, MA, USA) and ChemiDoc XRS (Biorad).

Through the same approach we also characterised α -synOs at the final concentration of 1, 5, 10 μ M. In this case we run 0.5 μ g for each sample.

5.6 Alpha-synOs and vehicle ICV injection

Monomeric, oligomeric or fibrillar α -syn and Veh were ICV injected (1 μ L/min) through an injection unit inserted inside the previously implanted guide cannula and connected to a Hamilton syringe with a flexible plastic tubing, sufficiently long to allow mouse free movements within their own cages. At the end of injection, the injection unit was removed

two minutes after drug delivery to allow its diffusion. Control mice were injected with the corresponding volume of Veh. To assure the absence of adverse effects due to the ICV injection, at the end of the ICV administration mice were monitored in their own homecage.

5.7 Drugs and treatments

- *Veh* (50mM PBS), α -syn monomers, oligomers or fibrils were ICV injected (as described above and detailed in each result section) using a Hamilton syringe connected to an infusion pump (1 μ L/minute).

-*Indomethacin* (Indo; Sigma-Aldrich) dissolved in 0.2M Tris-HCl, pH 8.2 was injected at 10mg/Kg intraperitoneally (IP) 30 minutes before α -synOs or Veh administration.

-*Ibuprofen* (IBF; Sigma-Aldrich) was dissolved in 100mM PBS and IP injected at 5, 10 and 50mg/Kg 30 minutes before α -synOs or Veh administration.

-*4G8* and *anti- α -syn antibodies* (Signet and BD Trasduction Laboratories respectively) were ICV injected at the dose of 0.25 μ g/2 μ L 15 minutes before α -synOs injection.

-*Anti-TLR2 antibody (T2.5) (Invivogen)* was dissolved in PBS and ICV injected at the dose of 5 μ g/2 μ L 15 minutes before α -synOs injection.

All drugs were injected ICV through an injection unit inserted inside the implanted guide cannula and connected to the Hamilton syringe through a flexible plastic tubing, sufficiently long to allow mouse free movements within their own cages during injection. The injection unit was removed two minutes after drug delivery to allow diffusion. Control mice were injected with the corresponding volume of the appropriate vehicle.

-*Ultra-pure LPS* from *E. Coli* (strain 011:B4; InvitroGen, USA) was dissolved in sterile water and IP injected at 1 or 2.5 mg/Kg. For the *in vitro* treatment, LPS was used at the final concentration of 0.5 μ g/mL.

5.8 Novel object recognition task (NORT) and open field

The task started with a habituation trial (open field; five min) during which mice were placed in an empty arena (40x40 cm and 30 cm high) and they were free to explore the new environment. In the next day (sample phase; ten min) animals were placed in the arena with two identical objects and free to explore them. Twenty-four hours later, animals were replaced in the arena where a familiar object was replaced with a novel one (test phase; ten min). Time spent by mice to explore both the familiar and the novel object were measured by a blind operator.

The following objects were used: a black plastic cylinder (4 x 5 cm), a glass vial with a white cup (3 x 6 cm) and a metal cube (3 x 5 cm).

Memory was expressed as a discrimination index [DI=(seconds on novel-seconds on familiar)/total investigation time]. Animals with memory impairment spent the same time in investigating the familiar and the novel object giving a lower DI compared to animals without memory impairment.

5.9 Y-maze test

Short-term spatial memory was assessed in the Y-maze task (Xu *et al.*, 2018). The Y-maze apparatus is a Y-shaped maze with three 20 cm length grey arms at the angle of 120° from each other. Each arm is large 5 cm and has a high of 10 cm.

Mice were placed in the centre of the Y-maze apparatus and free to move for eight min. A successful alternation was defined as a mouse entering consequentially in three different arms of the maze (*i.e.* ABC, BAC, BCA but not ABA, CAC, BCB). The percentage of spontaneous alternation [number of successful alternations/(total entries-2)]*100 was calculated by a blinding operator. Since mice prefer to visit a new arm of the maze and show a tendency to enter the less recently explored arm, animals displaying a memory impairment

have lower spontaneous alternation behaviour than mice not impaired. Of note, an arm entry is considered when all the four mouse limbs are into the arm.

5.10 Morris-water maze (MWM) test

Learning and spatial memory were investigated through the MWM test. A circular arena (100 diameter x 50 cm height) was filled to a depth of 0.29 m with water at $21\pm 1^{\circ}\text{C}$. The water was made cloudy using a nontoxic white dye. A white platform (11x11 cm) was positioned in a specific quadrant (Target Quadrant) 0.5 cm below the water and animals were trained to find it through a 4-trial training (60 seconds/trial with a 10 minutes inter-trial interval) for five consecutive days. The escape latency to find the platform through days was measured and expressed as mean \pm SEM of the 4 trials. On day six, platform was removed (probe phase, 60 seconds) and mouse ability to recall the platform location was assessed measuring the time spent in both target and opposite quadrant.

5.11 Beam-walk test

The beam-walk test (Pischiutta *et al.*, 2018) was performed to evaluate mouse gait instability. Briefly, the beam-walk test measures the footslips of mouse walking twice on an elevated, wooden beam (8 mm wide and 100 cm long). Before test, mice are trained in three habituation trials. Data are expressed as the footslips mean \pm SEM in the two tests. Mice with gait instability done a higher footslips number than not impaired animals.

5.12 Open field

To assess the spontaneous motor behaviour, we have exploited the open field task. Mice were placed in an empty arena (40x40 cm and 30 cm high) for 5 minutes and they were free to explore the new environment. The spontaneous motor behaviour of each mouse was

recorded and, through the EthoVision program, both the total distance moved (mean \pm SEM) and the mean velocity (mean \pm SEM) were measured.

5.13 Extracellular field recordings

Extracellular field recordings were performed by Dr. Milica Cerovic (Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy). Briefly, coronal brain slices (350 μ m) were cut in ice-cold modified artificial cerebrospinal fluid (aCSF: 87 mM NaCl, 2.5 mM KCl, 1 mM NaH₂PO₄, 75 mM Sucrose, 7 mM MgCl₂, 24 mM NaHCO₃, 11 mM D-glucose, and 0.5 mM CaCl₂). Brain coronal slices were transferred into the incubating chamber, submerged in aCSF containing 130 mM NaCl, 3.5 mM KCl, 1.2 mM NaH₂PO₄, 1.3 mM MgCl₂, 25 mM NaHCO₃, 11 mM D-glucose, 2 mM CaCl₂ and constantly bubbled with 95% O₂ and 5% CO₂ at room temperature (RT). Slices were incubated for at least 1 hour before recording and in a submerged recording chamber, perfused with oxygenated aCSF at a constant rate of 2-3 ml/min at 28-30°C. The pre-incubation with α -synOs (200nM/90-120 minutes) was performed at RT.

Stimuli were delivered via a Constant Voltage Isolated Stimulator (Digitimer Ltd., Welwyn Garden City, UK) with a bipolar twisted Ni/Cr stimulating electrode. LTP was induced by a 4-theta-burst tetanus stimulation protocol (each burst consists of four 100 Hz pulses with a 200 ms inter-burst interval). Signals were amplified and filtered (low filter 10 Hz, high filter 3 kHz) by a DAM 80 AC Differential Amplifier (World Precision Instruments, Sarasota, FL), and digitised at 10 kHz with a Digidata 1322 (Molecular Devices, Foster City, CA). LTP recordings in which the amplitude of the presynaptic fibre volley changed by more than 20% were discarded.

5.14 Immunohistochemistry and immunofluorescence

Mice were anaesthetised with a mix of ketamine (1.75 mg/Kg) and medetomidine (1 mg/Kg) and transcardially perfused with 50 mM PBS pH 7.4 followed by chilled 4% paraformaldehyde in 50 mM PBS. Serial coronal hippocampal and SN sections (30 μ m) were collected in 100 mM PBS for the immunohistochemistry analyses of the astroglial marker (GFAP), the microglial marker (IBA1), and the tyrosine hydroxylase (Th). Briefly, slices were blocked for 1 hour at RT with an appropriate blocking solution (GFAP: 3% NGS, Triton 0.4% in PBS 100 mM; IBA and Th: 10% NGS, Triton 0.3% in PBS 100mM). Then, slices were incubated with mouse anti-GFAP antibody (1:3500, Millipore), rabbit anti-IBA1 antibody (1:1000; Wako) or mouse anti-Th antibody 1:500, Millipore) at 4°C overnight (ON). After incubation with the anti-mouse biotinylated secondary antibody (1:200; Vector Laboratories; 1 hour RT) or the anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories; 1 hour RT), the immunostaining was developed using the avidin-biotin kit (Vector Laboratories) and diaminobenzidine (Sigma, Italy). Image acquisition was done using the Olympus VS120-S6-FL-078. Analyses of GFAP-, IBA1- and Th-immunoreactivity have been performed with a homemade macros and image analyser and the Fiji software.

Immunofluorescence analyses have been performed to evaluate the spreading of ICV injected α -syn (monomers, oligomers and fibrils) and to asses the expression of GFAP, IBA1 and IL-1 β or the expression of the M1 pro-inflammatory marker CD16/32 and its co-localisation with IBA1 in the hippocampus.

- Immunofluorescence for α -syn

Serial coronal hippocampal sections (20 μ m) were collected in 100 mM PBS for immunofluorescence analysis of the injected human recombinant α -syn. Freely-floating brain slices (three/mouse) were blocked (10% NGS, Triton 0.3% in PBS 100mM) for 1 hour at RT. Then, sections were incubated with the primary mouse antibodies against human α -

syn (1:1000, ThermoFisher). Fluorescence was detected using anti-mouse secondary antibody conjugated with Alexa 594 (Molecular Probes).

- Immunofluorescence for IBA1, GFAP and IL-1 β

Serial coronal hippocampal sections (30 μ m) were collected in 100 mM PBS for triple-immunofluorescence analysis of astrocytes, microglial cells and IL-1 β . Freely-floating brain slices (3/mouse) were incubated at 4°C for 10 min in 70% methanol and 2% H₂O₂ in Tris-HCl-buffered saline (TBS, pH 7.4) followed by 30 minutes incubation in 10% foetal bovine serum (FBS) in 1% Triton X-100 in TBS. Sections were incubated first with the primary antibody against IL-1 β (1:200, Santa Cruz Biotechnology; 72 h, 4°C in 10% FBS/1% Triton X-100/TBS). Then slices were incubated with the biotinylated secondary anti-goat antibody (1:200, Vector Labs) followed by fluorescent signal coupling with a streptavidin Tyramide Signal Amplification kit (NEN Life Science Products). Sections were incubated with the primary antibodies against GFAP (1:3500; Millipore) and IBA1(1:1000; Wako) and fluorescence was detected using anti-mouse or anti-rabbit secondary antibody conjugated respectively with Alexa 546 or Alexa 695 (Molecular Probes).

- Immunofluorescence for CD16/32 and IBA1

Coronal hippocampal brain slices (three/mouse) were blocked (10% NGS, Triton 0.3% in PBS 100mM) for 1 hour at RT. Then, sections were incubated with the primary rat antibodies against CD16/32 (1:600, BD Pharmingen) and rabbit anti-IBA1 (1:1000; Wako).

Fluorescence was detected using anti-rat or anti-rabbit secondary antibody conjugated respectively with Alexa 647 or Alexa 546 (Molecular Probes). Immunofluorescence was acquired using an IX81 microscope equipped with a motorised stage and a FV500 confocal scan unit with three laser lines [argon-krypton (488 nm), helium-neon red (646 nm), and helium-neon green (532 nm; Olympus)] and an ultraviolet diode.

5.15 Nissl staining

Three coronal hippocampal brain slices for each sample were hydrated in distilled water (1 minute). Then, brain slices were dehydrated through an alcoholic scale (Ethanol 70%, Ethanol 95%, Ethanol 100% and Xilene; 5 minutes/phase). Brain slices were stained in 0.5% Cresyl violet (Santa Cruz Biotechnology) solution in 25% Methanol. The excessive staining was removed through 10 consecutive washes in distilled water and then fixed through the same inverse alcoholic scale described above (3 minutes/phase).

5.16 Western blotting of synaptic proteins

Mice (4-5/group) were anesthetised with a mix of ketamine (1.75 mg/Kg) and medetomidine (1 mg/Kg) then decapitated. The hippocampus was dissected, frozen on dry ice and stored at -80°C. Tissues were homogenised to obtain the total insoluble fraction (TIF) and the expression of PSD95 and synaptophysin assessed. Briefly, to obtain the TIF, tissues were homogenised in ice-cold lysis buffer (0.32 M sucrose containing 1 mM Hepes, 1 mM MgCl₂, 1 mM NaHCO₃, 0.1 mM, PMSF, at pH 7.4, with a complete set of protease inhibitors (Sigma). Total homogenate was centrifuged at 1000 g for 10 min at 4°C and the resulting supernatant was centrifuged again at 13000 g for 15 min. The pellet obtained was suspended in ipotonic buffer (HEPES 1mM and protease inhibitor cocktails) and centrifuged at 100000 g for 1 hour. Then, the pellet was re-suspended in Triton X-100 1% with KCl 150 mM and protein inhibitor cocktail. The suspension was centrifuged at 100000 g (4°C, 1 hour). The pellet obtained (TIF) was then suspended in a buffer containing Glycerol 30%, HEPES 1 mM and protein inhibitor cocktail.

Total protein content was measured in the TIF fraction by the Bio-Rad Protein Assay (Bio-Rad Laboratories). Gels (8% SDS-PAGE for PSD95 and Synaptophysin) were run under reducing conditions; 10 µg proteins from each sample were run in duplicate and nitrocellulose membranes obtained from electroblotting were probed using the following

antibodies: mouse anti-PSD95 (Neuromab, 1:5000 in non-fat milk/TBS 0.1% Tween-20) and rabbit anti-synaptophysin (Synaptic System, 1:5000 in 3% BSA/TBS 0.1% Tween-20) at 4°C ON. Then, membranes were incubated with the mouse or rabbit HRP secondary antibody (1:5000; RT 1 hour; Abcam). Immunoreactivity was visualised with Lumnia Forte Western HRP substrate (Millipore, Billerica, MA, USA) and ChemiDoc XRS (Biorad). Optical density of the blots was measured with Quantity-One software (Biorad) and normalised using the corresponding signal for β -actin (Santa Cruz Biotechnology). Results were expressed as fold change (%) of Veh-treated mice.

5.17 Hippocampal neuron cultures and determination of α -synO toxicity

Hippocampal neurons were prepared by Dr. Elena Restelli (Istituto di Ricerche Farmacologiche Marion Negri, Milano, Italy) as previously described (Restelli *et al.*, 2010). Briefly, Two-day-old mouse hippocampi were incubated in neuronal dissociation medium (5.8 mM MgCl₂, 0.5 mM CaCl₂, 3.2 mM HEPES, 0.2 mM NaOH, 30 mM K₂SO₄, 0.5 μ g/ml phenol red, pH 7.4; 292 mOsmol) containing 20 U/ml papain (Sigma-Aldrich) at 34°C for 30 minutes. Trypsin inhibitor (Sigma-Aldrich) was added to a final concentration of 0.5 mg/ml and the tissue was mechanically dissociated. Cells were plated at 200000-250000 cells/cm² on plates coated with poly-d-lysine (25 μ g/ml) and maintained in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), penicillin/streptomycin, and glutamine 2 mM. Cell viability was assessed measuring the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan previously described (or through the lactate dehydrogenase (LDH) assay. In the MTT assay cells were incubated at 37°C for 3 hours with 0.4 mg/ml MTT, dissolved in 0.04 N HCl in 2-propanol, and analyzed spectrophotometrically at 540 nm with an automatic microplate reader (Tecan Infinite M200). In the LDH assay, 50 μ L of cell medium were collected from each well at the end of treatments. 50 μ L of Cytotox96 Reagent (12 mL of Assay buffer in one vial of

Substrate mix) were added to the cell medium. The assay plate was mixed gently for 1 minute and incubated in the dark (30 minutes, RT). Following the incubation, 50 μ L of Stop Solution were added to the wells to interrupt the reaction and the absorbance was measured at 490 nm through automatic microplate reader (Tecan Infinite M200).

LDH positive control is provided in the LDH assay kit (CytoTox96 non r-radioactive cytotoxicity assay, Promega).

All treatments of neuronal hippocampal cultures started 12 days after neuronal plating. Details for each treatment were provided in each result section.

5.18 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) analyses were performed to assess the direct PrP^C- α -synO binding by Dr. Marten Beeg and Dr. Marco Gobbi (Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milano, Italy). α -synOs (1 and 10 M) or A β -Os (1 μ M) were injected over the surfaces exposing PrP^C captured by 3F4 or 94B4, or those coated with 3F4 or 94B4 alone (without PrP^C, used for reference). Assays were performed at 25 °C. The α -syn- or A β -dependent signals on the surfaces immobilizing PrP^C were obtained by double referencing, subtracting the response observed on surfaces immobilizing the antibodies alone, and the signal observed injecting the Veh alone (which allows correction for binding-independent responses, e.g., drift effects). The ProteOn XPR36 Protein Interaction Array system (Bio-Rad) was used. More in detail, anti-PrP^C monoclonal antibodies 3F4 and 94B4 were immobilised by amine-coupling chemistry on the surface of a GLC sensor chip (BioRad), according to manufacturer recommendations. The final immobilisation levels were ~6,000 resonance units (1 RU = 1 pg protein/mm²) for both 3F4 and 94B4. “Reference” surfaces were prepared in parallel following the same immobilisation procedure but without antibodies. Then, total brain homogenate (0.5 mg protein/mL in PBS containing 0.5% Nonidet P-40 and 0.5% Na-deoxycholate) from WT-E1 Tg mice overexpressing WT

mouse PrP carrying an epitope tag for the monoclonal antibody 3F4 (Chiesa *et al.*, 1998) were flowed on the surface of chips. While the signal was negligible in the “reference” surface after the flowing of total brain homogenate, it was ~900 on the surfaces coated with 3F4 and ~700 RU on the surfaces coated with 94B4. Since the signal decayed very slowly, the dissociation of PrP^C from both 3F4 and 94B4 is slow.

5.19 Statistical analysis

Data were analysed using the GraphPad Prism 7.0 software. Unpaired Student’s T-test, One-, Two- or Three-way analyses of variance (ANOVA) were used for the statistical analyses of our experiments in accordance with the amount of the independent variables (factors/treatments) which affect the dependent variable (*e.g.* the DI, the % of spontaneous alternation, the % of GFAP- and IBA1-marked area...) as specified in each result sections. In presence of a significant effect of *treatment* in the One-way ANOVA or a significant *interaction* among variables in the Two- and Three-way ANOVA, an appropriate post-hoc tests was applied (details in each result sections). For all analyses, a p value < 0.05 was considered statistically significant.

Results

Chapter VI

***Alpha-synOs acutely impair memory whereas
monomers and fibrils were ineffective***

The data presented in this chapter are reported in the manuscript entitled “Alpha-synuclein oligomers impair memory through glial cell activation and via Toll-like receptor 2”. *Brain Behav Immun.* 2018 Mar; 69:591-602. doi: 10.1016/j.bbi.2018.02.012.

6.1 Aim of the study and *in vivo* experimental design

Based on the evidence previously reported, in this section we aimed to specifically investigate the α -syn oligomeric hypothesis in the context of memory impairment. To this purpose, we have developed an acute mouse model based on the ICV injection of monomers, oligomers or fibrils of α -syn, and we have evaluated their abilities to cause a memory damage in the NORT. Of note, unlike Tg PD mouse models, where different α -syn aggregates coexist, the use of the acute mouse model specifically allowed us to assess the detrimental effects on memory of each extracellular moieties individually, and to characterise this effect at behavioural as well as histological level.

Precisely, two hours before the familiarisation/sample phase of the NORT, C57BL/6 naïve mice were ICV injected with Veh or well-characterised solutions enriched in α -syn monomers, oligomers or fibrils. The memory performance of each mouse was assessed in the test phase 24 hours later (Figure 19A). Thus, an inter-trial time of 24 hours between the familiarisation/sample phase and the test phase occurs, such that it allows us to accurately test the effect of each moiety on long-term recognition memory. Notably, more than 8 hours are required to permit consolidation of long-term memory (Sutton and Schuman, 2006), and the 24 hours is the most widely used time point for its evaluation (Balducci *et al.*, 2010; Balducci *et al.*, 2017). To further characterise the model, we investigated whether the α -synO-induced memory damage was specifically mediated by α -syn. To this goal two different antibodies, the A β O specific antibody 4G8 and the Anti- α -syn antibody (ICV, 15 minutes pre- α -synOs), were tested for their ability to counteract the α -synO-triggered memory damage (Figure 19B). Once demonstrated that α -synOs specifically impaired

memory performance in the NORT, we further characterised such a detrimental effect. Thus, we assessed whether the cognitive impairment was persistent or transient. To address this question, mice treated with α -synOs impaired in their memory performance were re-tested in the NORT 12 days after the first ICV injection without any other treatment (Figure 19C). For a detailed description of drug doses see chapter V about methods.

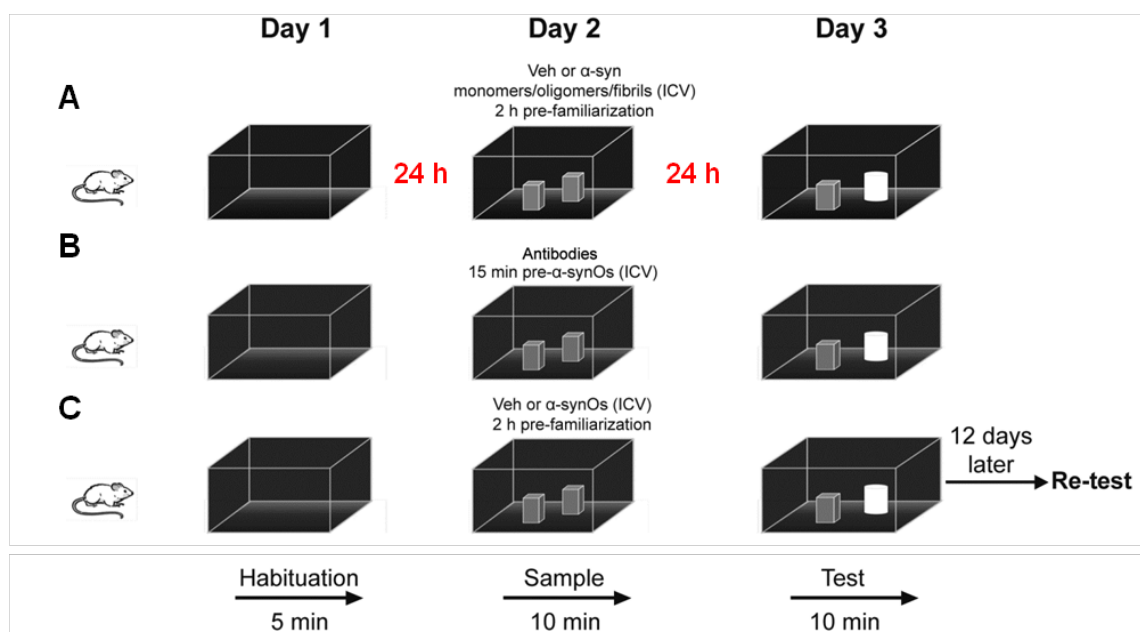


Figure 19. Schematic of treatment schedules for NORT experiments. (A) Monomers, oligomers fibrils of α -syn ($1\mu\text{M}/7.5\mu\text{L}$) ICV injected 2 hours before the familiarisation/sample phase. **(B)** Treatment with 4G8 or Anti- α -syn ($0.25\mu\text{g}/2\mu\text{L}$) 15 minutes pre- α -synOs injection ($1\mu\text{M}/7.5\mu\text{L}$). **(C)** Mice re-tested in NORT without any further ICV injection of Veh or α -synOs.

6.2 Results

6.2.1 Alpha-synOs specifically induce memory deficiency in C57BL/6 naïve mice and impair hippocampal LTP on brain slices

Consistent with the oligomeric hypothesis which pinpoints α -synOs as the main culprits underlying α -syn deleterious effects, oligomeric species of α -syn have been demonstrated to impair LTP and cause memory damage (Martin *et al.*, 2012).

To further assess the detrimental effects of α -synOs on memory in comparison with either α -syn monomers or fibrils, we have developed an acute model based on a single ICV injection of well-characterised solutions enriched in monomeric, oligomeric or fibrillar α -syn (7.5 μ L, 1.0 μ M) in C57BL/6 naïve mice. Endotoxin-free recombinant α -syn monomers (Figure 20A) were provided by Pollegioni's team (University of Insubria, Varese, Italy) which also characterised the protein and demonstrated the partial random coil structure of the protein in solution through far-UV circular dichroism analysis (Caldinelli *et al.*, 2013). α -synOs and fibrils used for the experiments were characterised by AFM to verify their aggregation status and their distribution in terms of diameter and height (Figure 20B-F). In addition, the α -synO solution was further characterised through western blot, which confirmed an enrichment in SDS-resistant oligomeric species with a molecular weight ranging from 35 to 180 KDa (Figure 20G).

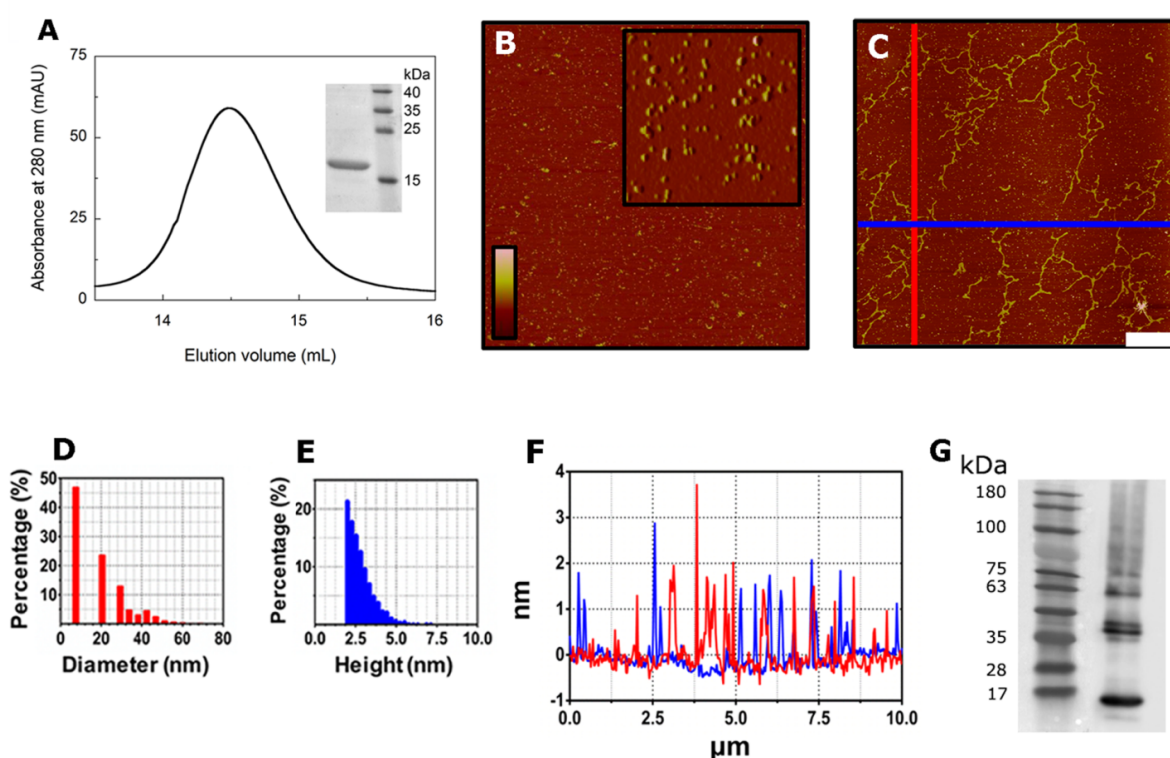


Figure 20. Native size-exclusion chromatography and tapping mode AFM images. (A) Elution profile of purified recombinant α -syn by Superdex-200 column chromatography and SDS-PAGE analysis of the eluted protein. (B) α -synO assemblies after incubation for 48 hours, or (C) α -syn fibres after 5 days incubation in 50 mM PBS. Scale bars correspond to 2.5 μ m for Panels B and C,

and 0.5 μm for the Panel B inset. The scale colours correspond to height range of -5/+10 nm for Panel B, -30/+50 nm for Panel B inset and -2/+4 for Panel C. **(D, E)** Oligomer distribution in diameter and height. **(F)** Cross-sectional profiles determined by SPIP and same colour code used to represent the regions of images chosen for measurements and their line-profile in samples incubated for 5 days. **(G)** Western blot analysis of α -synO-enriched solution showing SDS-resistant oligomers with a molecular weight ranging from 35 to 180 KDa.

Once verified the nature of our preparations, mice were ICV injected with Veh (7.5 μL), monomeric, oligomeric or fibrillar α -syn (1 μM /7.5 μL) 2 hours before the sample phase of the NORT, and memory was investigated 24 hours later. The behavioural assessment of long-term recognition memory revealed a significant effect of *treatment* ($F_{3,41}=3.153$, $P=0.03$; One-way ANOVA). In particular, we found that mice receiving Veh, monomers or fibrils discriminated well between the novel and the familiar object. In fact, as shown in figure 21A, they spent more time investigating the novel object than the familiar one, as also demonstrated by a comparable DI (mean \pm SEM) between the three experimental groups (0.23 ± 0.06 ; 0.23 ± 0.06 and 0.22 ± 0.04 respectively). In contrast, α -synO-treated mice did not discriminate between the two objects. Indeed, they spent an equal time on both the familiar and the novel one, and they showed a significantly lower DI than Veh-treated mice (0.23 ± 0.06 and 0.002 ± 0.08 respectively; $*p<0.05$, One-way ANOVA followed by Dunnet's test; Figure 21A, B). Of note, it could be speculated that the memory deficiency in α -synO-treated mice may arise from a reduced exploratory behaviour rather than an effect of α -synOs on memory. Thus, such an effect of α -synOs could make treated mice unable to recognise the familiar object during the test day. To check whether this bias affected our experimental conditions, we compared the total exploration time spent investigating the objects during both the sample and the test phase for each experimental group (Figure 21C). As the total exploration time during the two NORT phases did not significantly differ between groups, as demonstrated by the One-way ANOVA which did not find significant differences among groups for both the sample and the test phases (Sample phase: $F_{3,41}=0.52$ $P=0.67$; Test phase:

$F_{3,41}=1.55$, $P=0.21$), we ruled out that the memory impairment triggered by α -synOs was due to a lower object exploration. To be noticed that the lower exploratory behaviour during the test phase of mice treated with α -syn fibrils ($n=11$) compared to Veh-treated mice ($n=11$) was due to the presence of two mice in the fibrils-treated group that explored the objects for less than 10 seconds. Aside from these mice, the total exploratory behaviour of the fibrils-treated group during the test phase became comparable to the Veh-treated group (20 ± 3.4 and 24 ± 2.9 sec respectively). Moreover, although these two mice spent a less time in investigating the objects, they did not show a memory impairment having a DI higher than 0.20 and during these experiments animals did not displayed an impairment of their motor performances in the arena.

To further characterise the α -synO-mediated memory damage, we ascertained whether recognition memory was specifically impaired by α -syn. To this aim, we ICV pre-treated mice with either a specific anti α -syn antibody or the specific anti-A β 4G8 antibody with the same dose of 0.25 μ g. As shown in figure 21D, 4G8 pre-treatment did not abolish the effect of α -synOs. We found indeed that mice receiving either α -synOs alone or with 4G8 still exhibited a significant impairment of their memory performance (DI=-0.12 \pm 0.09 and DI=0.01 \pm 0.04 respectively; ** $p<0.01$, One-way ANOVA followed by Tukey's test) compared to Veh-treated mice (DI=0.25 \pm 0.04). Conversely, the anti- α -syn antibody pre-treatment fully abrogated the α -synO-mediated memory deficiency. In fact, mice receiving α -synOs alone had a significantly lower DI than mice pre-treated with the anti- α -syn antibody (0.02 \pm 0.07 and 0.22 \pm 0.07 respectively; * $p<0.05$, One-way ANOVA followed by Tukey's test). Notably, mice receiving the anti- α -syn antibody+ α -synOs showed a memory performance comparable to Veh-treated mice (DI=0.22 \pm 0.07 and DI=0.27 \pm 0.05; Figure 21E).

Because of the α -synO-mediated memory impairment, and the previous reported ability of oligomers to impact on LTP (Diogenes *et al.*, 2012; Martin *et al.*, 2012; Ferreira *et al.*, 2017),

we investigated whether our oligomeric preparation could affect this process as well. Mice coronal brain slices pre-incubated for 60-90 minutes with α -synOs (200 nM) were used in experiments of extracellular field recordings of field excitatory postsynaptic potential (fEPSPs) in the CA1 hippocampal region evoked by stimulation of Schaffer collaterals. Through this approach, we found that α -synOs significantly reduced LTP (Figure 21F, G). Thus, we demonstrated that oligomeric α -syn ICV injected in mice leads to a detrimental effect on memory, whereas monomeric and fibrillar α -syn was ineffective. In addition, the memory damage we described is not linked to an altered exploratory behaviour of treated mice and it is specifically due to α -syn. Consistent with the memory impairment and previously reported data, α -synOs affect also the LTP, which is crucial for learning and memory. Thus, our findings corroborate the α -syn oligomeric hypothesis, and our acute model provides a valuable tool to specifically dissect α -synO detrimental activities on memory.

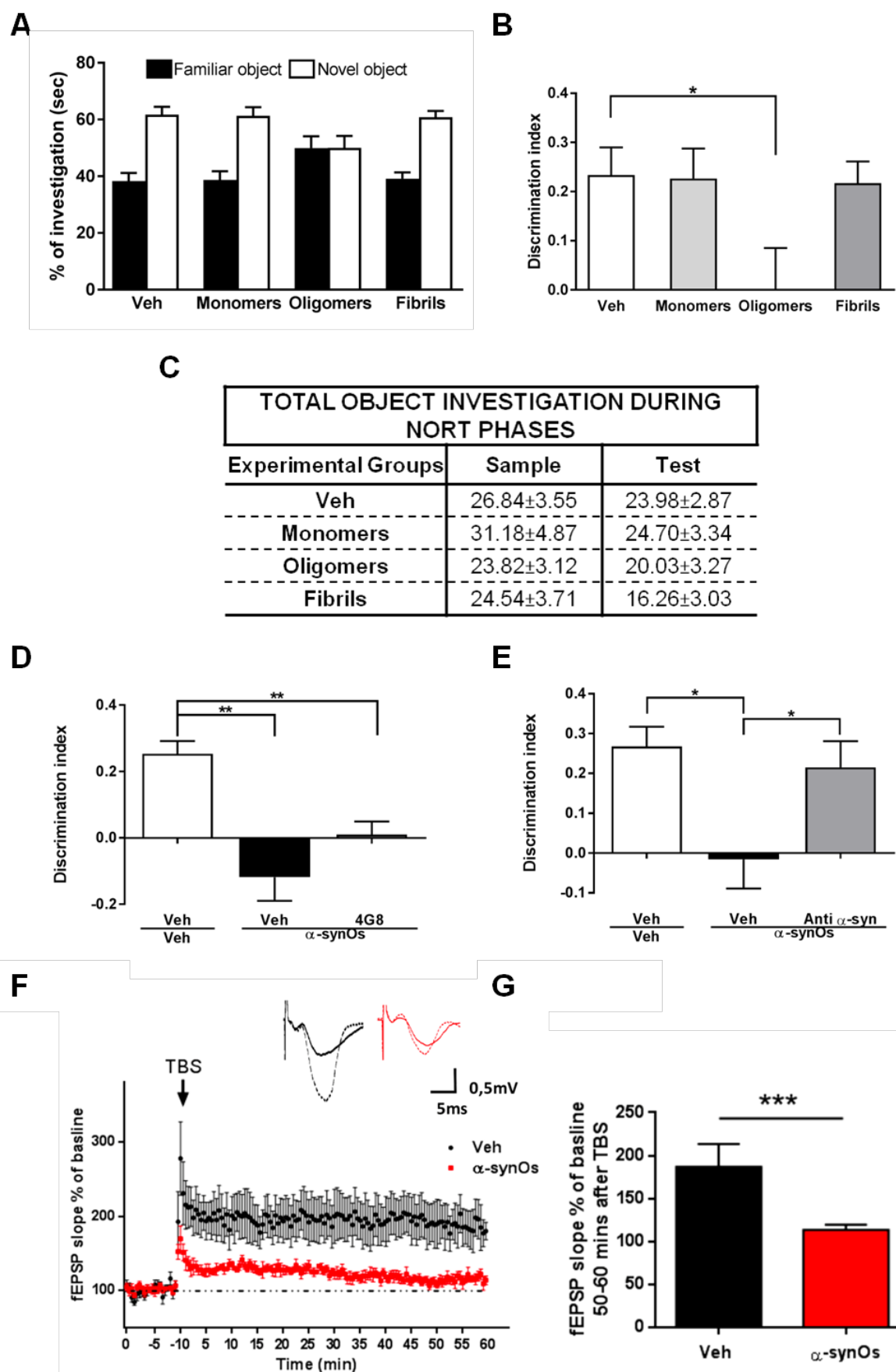


Figure 21. A single ICV injection of α -synOs impairs recognition memory and inhibits LTP in brain slices. (A) Histograms are mean±SEM percentage of time spent investigating the familiar and

novel object in each experimental group tested in the NORT after treatment with: Veh (n=12), α -syn monomers (n=11), α -synOs (n=11) or α -syn fibrils (n=11). **(B)** Histograms are the mean \pm SEM of the corresponding DI; One-way ANOVA found a significant effect of *treatment*: $F_{3,41}=3.1$, $P=0.03$. (* $p<0.05$, Dunnett's test). **(C)** Mean \pm SEM of total time (sec) spent in the exploration of the objects during the familiarisation phase (Sample), and during the test phase (Test) in the different experimental groups. One-way ANOVA found no effect of *treatment* for both the sample and test phase ($F_{3,41}=0.52$, $P=0.67$; $F_{3,41}=1.55$, $P=0.21$ respectively). **(D)** 4G8 treatment (0.25 μ g/2 μ L) 15 minutes before the ICV injection of α -synOs did not abolish α -synO-mediated memory impairment. Histogram are mean \pm SEM of the DI (Veh+Veh; Veh+ α -synOs and 4G8+ α -synOs; n=9/group; ** $p<0.01$, Tukey's test). One-way ANOVA found a significant effect of *treatment* ($F_{2,24}=13.4$, $P=0.0001$) due to the significant impairment induced by α -synOs and the inability of 4G8 to abolish it. **(E)** Effect of Anti- α -syn (0.25 μ g/2 μ L) 15 minutes before the ICV injection of α -synOs. Histograms are the DI mean \pm SEM of mice receiving Veh+Veh (n=9), Veh+ α -synOs (n=8) and Anti- α -syn+ α -synOs (n=8). One-way ANOVA found a significant effect of *treatment* ($F_{2,22}=5.8$, $P=0.0096$; * $p<0.05$, Tukey's test). **(F)** LTP in CA1 hippocampal region of Veh- (n=5) versus α -synO-treated slices (200nM α -synOs 90 min pre-incubation; n=5; $p<0.05$, Two-way ANOVA for repeated measures). Data are presented as time courses (mean \pm SEM) of normalised fEPSP slopes. Insets are representative traces of fEPSPs before (full line) and after TBS (dashed line), in control (black) and α -synOs treated slices (red). **(G)** fEPSP slope 50-60 min post theta-burst stimulation shown as a % (mean \pm SEM) of pre-stimulation baseline; (***) $p<0.001$, Student's t-test).

6.2.2 ICV injected α -syn spreads across the hippocampus

Our behavioural experiments demonstrated an impaired hippocampal-dependent memory establishment in the NORT caused by the ICV injection of α -synOs exclusively, whereas monomers and fibrils were ineffective. Therefore, we have investigated the ability of each moieties to spread from the injection site to the hippocampal parenchyma. As shown in the immunofluorescence analysis in Figure 22, monomeric, oligomeric and fibrillar moieties of α -syn were detectable in the hippocampus 4 hours after the ICV injection. Thus, our observation suggests that either the detrimental effects of α -synOs on memory, or the ineffectiveness of α -syn monomers/fibrils were not biased from the inability of our aggregates to spread within the hippocampus.

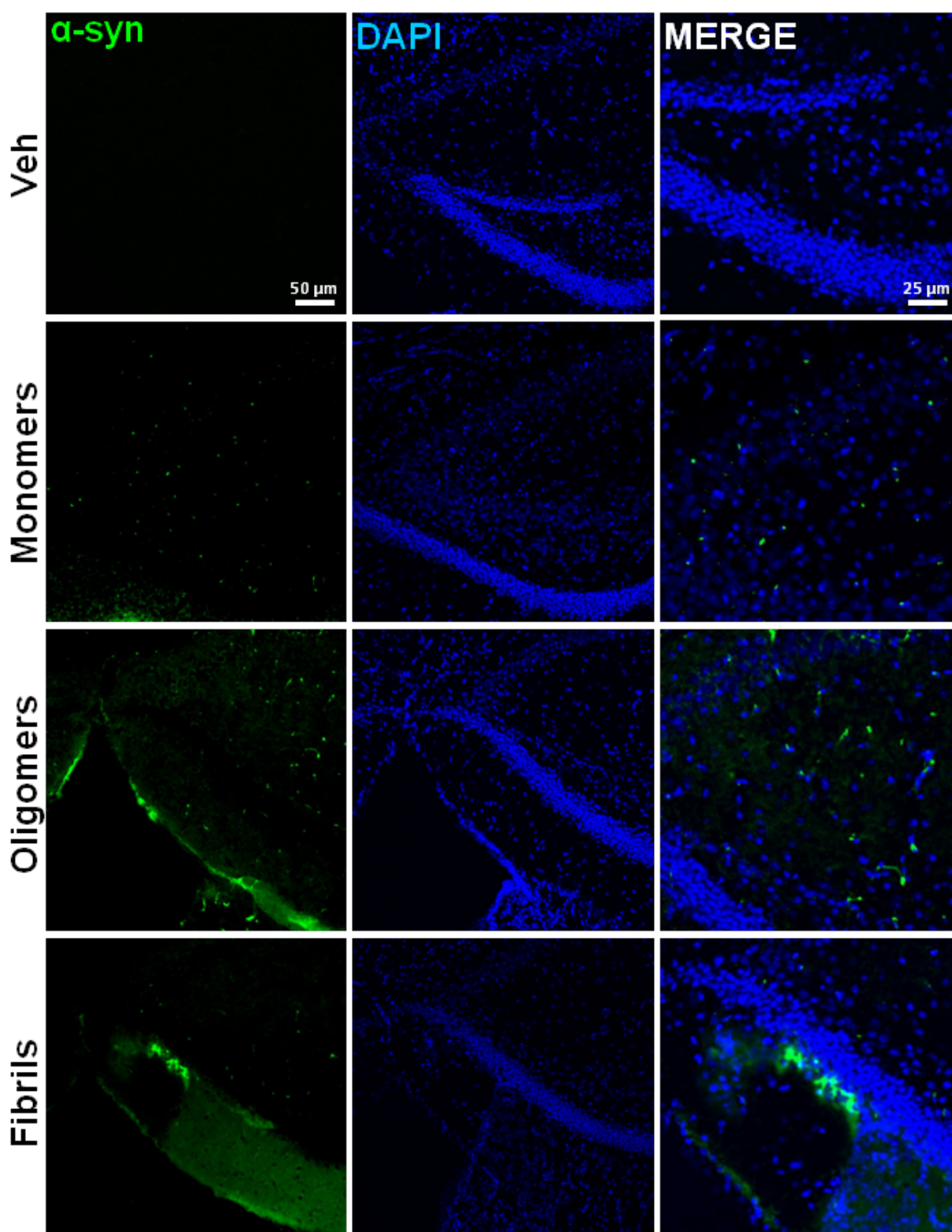


Figure 22. ICV injected α -syn moieties spread across the hippocampal parenchyma. Representative images of hippocampal section stained with an antibody against human α -syn showing green spots of different size across the hippocampus 4 hours after the ICV injection of α -syn monomers, oligomers and fibrils.

6.2.3 Alpha-synO-mediated recognition memory impairment is transient and not associated with hippocampal alterations at both neuronal and synaptic level

To further characterise the α -synO-mediated memory damage, we assessed whether this effect was permanent or transient. To this aim, we re-tested α -synO-injected mice which previously displayed an impairment in their recognition memory (Veh: DI=0.21 \pm 0.04 and α -synOs: DI= 0.02 \pm 0.07; t_{16} =2.24, *P=0.03) 12 days later, with no further injections.

As shown in figure 23A, mice previously receiving α -synOs performed well in the NORT, having a DI comparable to previously Veh-treated mice (0.23 \pm 0.06 and 0.29 \pm 0.06 respectively; t_{16} =0.81, P=0.43; Figure 23A).

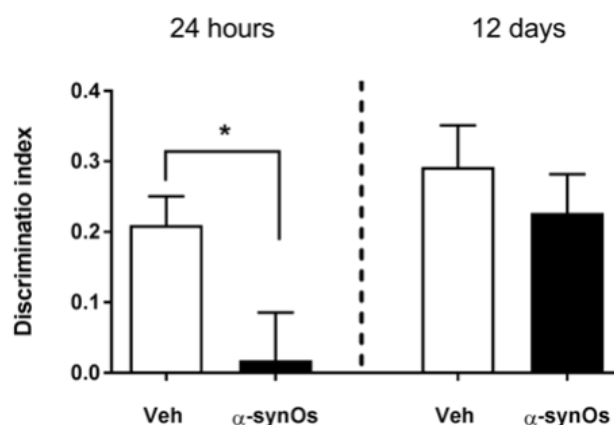


Figure 23. A single ICV injection of α -synOs transiently impairs recognition memory establishment. Histograms are mean \pm SEM of the corresponding DI at both 24 hours and 12 days after the first ICV injection of Veh or α -synOs (n=9/group).

Notably, the transient nature of the α -synO harmful effects on memory was consistent with the absence of significant alterations at both cellular organisation and synaptic protein level in the hippocampus of treated mice. In fact, we investigated whether the α -synO-mediated memory impairment was triggered by hippocampal changes in cellular organisation or synaptic structure.

Two, 4, 8 or 24 hours after α -synO injection mice were sacrificed. By means of a qualitative analysis of Nissl stained brain slices we did not find any difference in the neuronal

organisation and density in the hippocampal CA1, CA2, CA3 and dentate gyrus of α -synO-treated mice at any time point considered compared to Veh-treated animals (Figure 24).

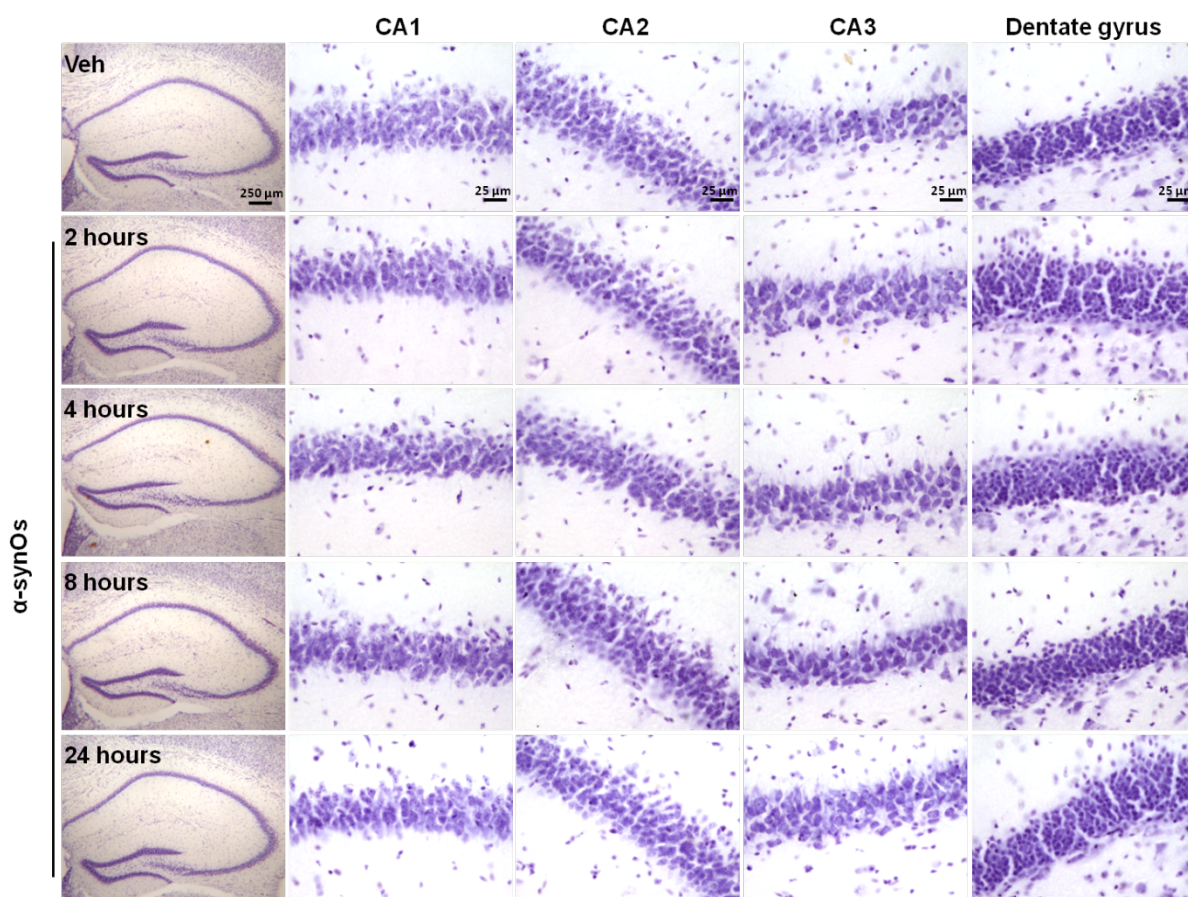


Figure 24. A single ICV injection of α -synOs does not alter neuronal organisation in the hippocampus. Representative images of Nissl staining coronal hippocampal sections and magnification of different hippocampal subfield 2, 4, 8 and 24 hours after α -synOs (3/group).

Moreover, we did not find any changes in the expression of representative pre- and post-synaptic proteins such as synaptophysin and PSD-95 at either 4- or 24-hours post-injection (Figure 25A-F). In fact, analysis of the optic density for both synaptophysin and PSD 95 had demonstrated the absence of significant differences between Veh- and α -synO-treated mice (PSD95 4 hours: $t_8=0.12$, $P=0.89$; PSD95 24 hours: $t_8=1.03$, $P=0.33$; synaptophysin 4 hours: $t_8=0.26$, $P=0.82$ and, synaptophysin 24 hours: $t_8=1.07$, $P=0.31$).

Together, our findings demonstrate that α -synOs lead to a transient memory impairment which is most likely dependent on a synaptic dysfunction rather than synaptic and neuronal loss.

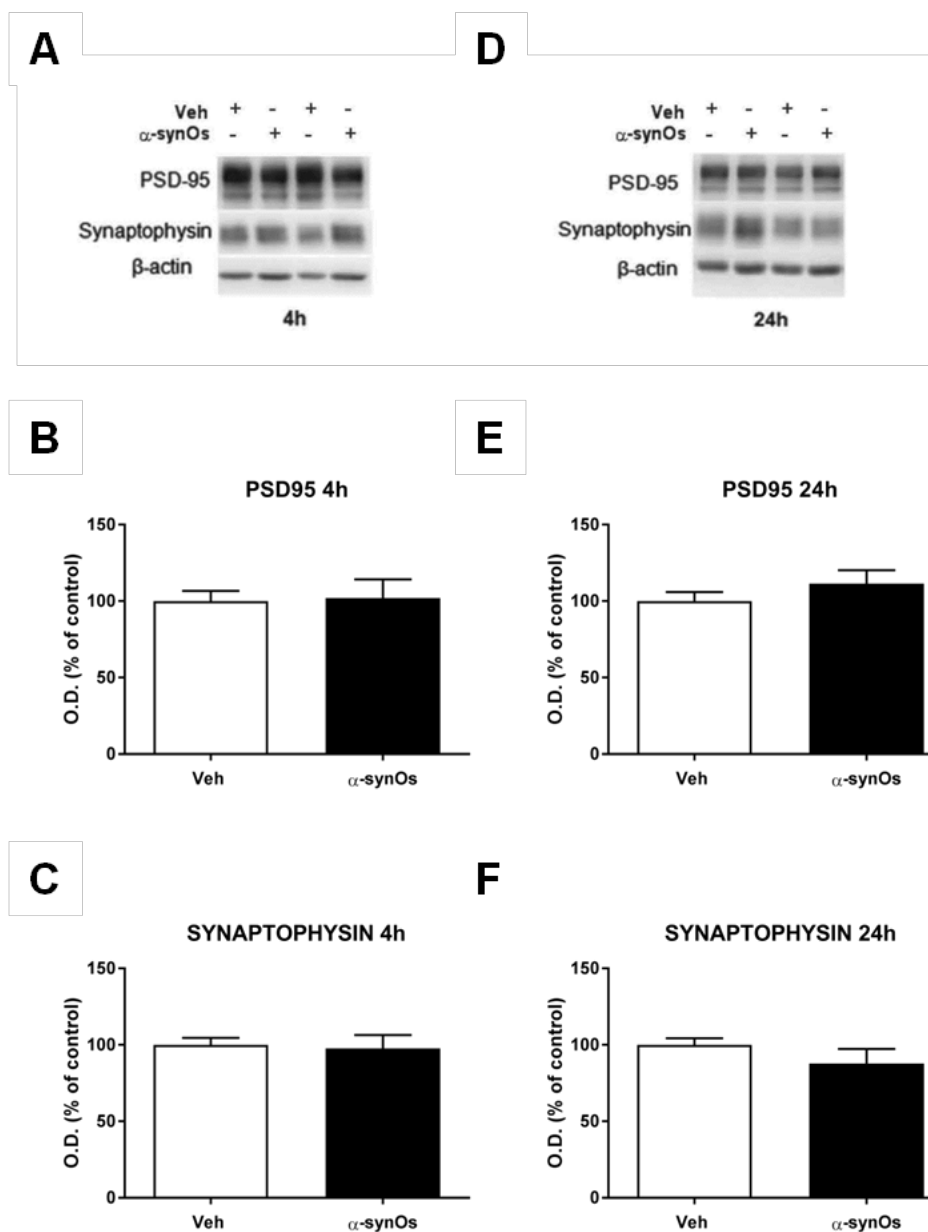


Figure 25. A single ICV injection of α -synOs does not alter synaptic markers in the hippocampus. (A-F) Immunoblot analysis for ipsilateral hippocampal (5 mice/group) PSD95 and synaptophysin expression, and relative quantification at both 4 and 24 hours. As demonstrated by the statistical analyses α -synOs do not affect the protein levels of either PSD95 or synaptophysin in each time point considered.

6.3 Discussion

Compelling evidence highlights α -syn as the main factor in the pathogenesis of PD and PD-related disorders. In pathological conditions, aggregation of α -syn leads to the production of small, soluble aggregates, namely oligomers, protofibrils and fibrils. α -synOs are heterogeneous and can vary in composition, structure and toxicity (Roberts and Brown, 2015). Consistent with the heterogeneous toxic profile of α -synOs, they are not all recognised as deleterious because hypothetically some of them might exert physiological functions, and furthermore the detrimental effects on neurons may be different upon exposure to different oligomeric conformations (Danzer *et al.*, 2007; Roberts and Brown, 2015). Regardless such a variability, the main accepted hypothesis in the field posits that α -synOs can be secreted by neurons and could be the main detrimental species involved in the pathogenesis of α -synucleinopathies, leading to cognitive dysfunction (Martin *et al.*, 2012; Diogenes *et al.*, 2012; Forloni *et al.*, 2016; Ono, 2017), which is commonly found among PD and LBD patients.

To test the “oligomeric hypothesis”, we took advantage of an acute mouse model based on a single ICV injection of different well-characterised α -syn moieties (monomer, oligomers, fibrils) in C57BL/6 naïve mice. Treated mice were tested for their memory in the NORT. We demonstrate that a single ICV injection of a solution enriched in α -synOs specifically caused a significant impairment in long-term memory. In contrast, monomers and fibrils were ineffective. The memory impairment we describe is specifically triggered by α -syn. In fact, while pre-treatment with an anti- α -syn antibody completely prevented the memory damage, pre-treatment with 4G8, which does not recognise α -syn but binds to oligomeric assemblies of A β (Stravalaci *et al.*, 2012), was inactive.

The memory deficit was not associated with neuronal loss or disorganisation in hippocampal cell layers. Similarly, no changes were detected in the expression of representative synaptic proteins. In fact, we did not find alterations of pre- and post-synaptic markers such as

synaptophysin or PSD95. In line with the absence of hippocampal structure alterations, we demonstrate that 12 days after the ICV injection of α -synOs, mouse memory was restored and comparable to the Veh-treated mice. Thus, in our acute mouse model, α -synOs lead to a transient rather than a persistent memory impairment.

To further address the detrimental action of α -synOs on memory, we investigated their effect on LTP, an experimental paradigm for studying the synaptic plasticity, which is a vital process in the consolidation of new memories. In fact, the absence of changes in synaptic structure would not rule out the occurrence of synaptic dysfunction. In agreement with previous reported data (Diogenes *et al.*, 2012; Martin *et al.*, 2012; Ferreira *et al.*, 2017), electrophysiological recordings confirmed that pre-incubation of brain slices with α -synOs severely reduces the amplitude of LTP. In addition, while we clearly depict α -synOs as the sole moieties leading to memory impairment in mice, we rule out the effectiveness of α -syn monomers and fibrils in the same process.

Results

Chapter VII

***Alpha-synO-mediated memory impairment is
dependent on glial activation and TLR2***

The data presented in this chapter are reported in the manuscript entitled “Alpha-synuclein oligomers impair memory through glial cell activation and via Toll-like receptor 2”. *Brain Behav Immun.* 2018 Mar; 69:591-602. doi: 10.1016/j.bbi.2018.02.012.

7.1 Aim of the study and experimental design

In the following section we sought to address the role of neuroinflammation in mediating the harmful activities of α -synOs on memory. In fact, despite initially considered as a mere secondary event, neuroinflammation has acquired an increasing interest in the pathogenesis of α -synucleinopathies.

Astrocytes and microglial cells are the main cellular components within the CNS. In spite of their different embryogenic origin, both astrocytes and microglial cells are immune-competent cells in the brain, and they play essential functions in maintaining brain homeostasis (Hertz and Chen, 2016). In healthy brains, astrocytes and microglial cells are juxtaposed to neurons and directly contact synapses at both pre- and post-synaptic level. This way, they are strictly involved in the surveillance of synaptic function and synaptic plasticity, which is a key player in governing learning and memory (Blank and Prinz, 2013). In response to various stimuli, including α -synOs, they become activated and able to release several pro-inflammatory factors which may trigger neuronal dysfunction (Fellner *et al.*, 2013; Kim *et al.*, 2013; Morris *et al.*, 2013; Blank and Prinz, 2013; Ramikko *et al.*, 2015; Kim *et al.*, 2016).

As a consequence, the uncontrolled activation of glial cells might deprive neurons of regulatory surveillance, and lead to impairment of synaptic plasticity and cognitive functions (Morris *et al.*, 2013; Richwine *et al.*, 2009).

As described in chapter III, initiation of an inflammatory response generally occurs after the recognition of DAMPs or PAMPs by PRRs such as TLRs. TLRs have been widely investigated in the context of PD (Kouli *et al.*, 2019). In particular, as previously described,

TLR2 and TLR4 have been identified as potential interactors for α -synOs. However, the precise contribution of the two receptors in mediating α -synO detrimental effects still remains unknown and a matter of debate (Kouli *et al.*, 2019).

Based on the evidence reported here, and on the gap of knowledge about the TLRs involvement in mediating α -synO detrimental effects, we herein aimed at: I) assessing whether α -synOs are capable of activating microglial and astroglial cells in the hippocampus in our acute mouse model; II) evaluating the contribution of the α -synO-induced neuroinflammation in triggering the memory damage as reported in chapter VI; and III) investigating the role of both TLR2 and TLR4 as mediators for α -synO-induced memory damage to get new insights on the mechanisms underlying α -synO detrimental effects.

To assess the presence of α -synO-induced astrogliosis and microgliosis in our acute mouse model, C57BL/6 naïve mice were ICV injected with α -synOs (1 μ M/7.5 μ L) or Veh and sacrificed at different time points (2, 4, 8, 24 hours) post-treatment. Then, activation of both astroglial and microglial cells in the hippocampus was investigated through immunofluorescence.

Once confirmed that the ICV injection of α -synOs was able to trigger a neuroinflammatory response, we addressed whether the α -synO-induced neuroinflammation was involved in mediating the memory damage taking place in α -synO-treated mice. To this purpose, we tested whether two different anti-inflammatory drugs (Indo and IBF, IP injected 30 minutes before α -synO ICV injection) could counteract the α -synO-induced memory deficiency (Figure 26A). In addition, through immunohistochemical approaches we assessed the activation state of astroglial and microglial cells in the hippocampus of mice treated with the anti-inflammatory drugs and α -synOs. Of note, assessment of the activation of glial cells was performed at the time point corresponding to the peak of glial activation (4 hours post α -synO ICV injection as described in the results). To get new insight into the role of TLRs in mediating α -synO detrimental effects on memory, we investigated the role of TLR4 and

TLR2. Specifically, TLR4 knock-out mice (TLR4^{0/0}) and WT mice were ICV injected with either α -synOs or Veh, and their memory performance tested in the NORT (Figure 26B). Consistent with the large body of evidence reporting the involvement of TLR2 in the pathogenesis of PD and PD-related disorders (Kim *et al.*, 2013; Kim *et al.*, 2016; Kim *et al.*, 2018), we also assess the role of TLR2 in mediating α -synO-induced memory deficiency in our acute mouse model. To this goal, we ICV pre-treated C57BL/6 naïve mice with the functional blocking antibody against TLR2 (T2.5) 15 minutes before α -synO or Veh injection (Figure 26C). For a detailed description of drug doses please see chapter V.

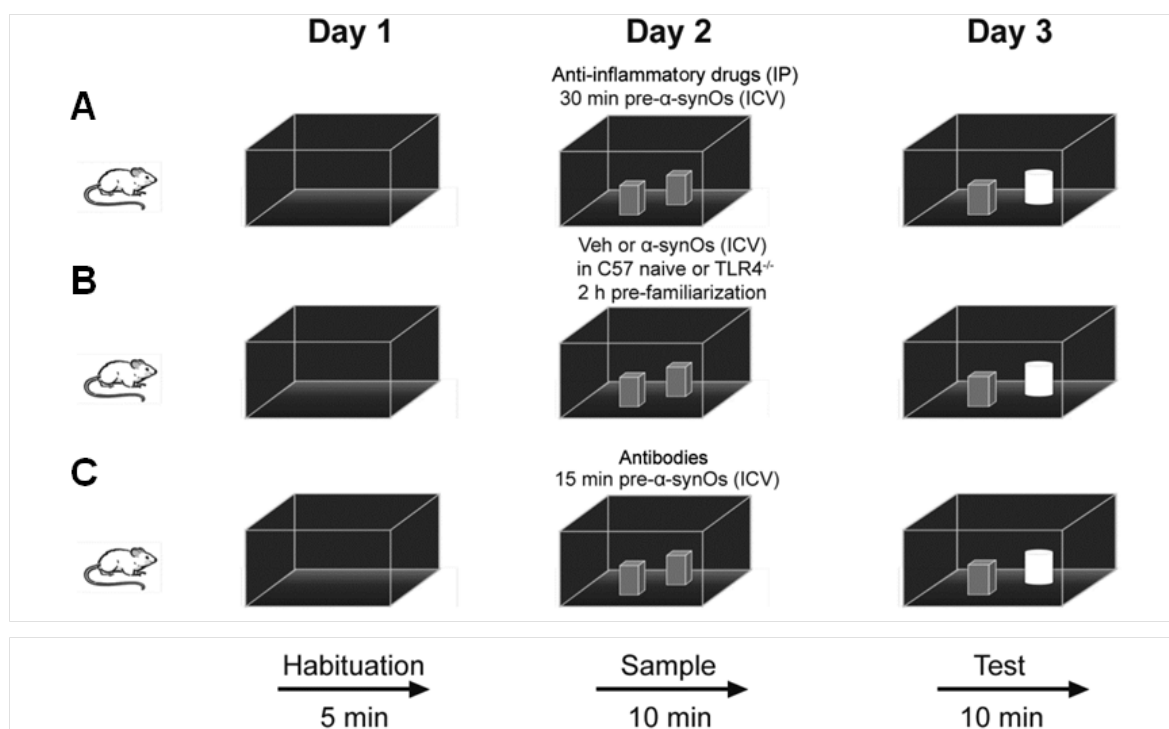


Figure 26. Schematic representation of treatment schedules for NORT experiments. (A) IP treatment with Indo (10mg/Kg) and IBF (5,10 and 50mg/Kg) 30 minutes before the ICV injection of α -synOs (1 μ M/7.5 μ L). (B) α -synOs treatment (1 μ M/7.5 μ L) in C57BL/6 naïve mice and TLR4^{0/0} mice. (C) ICV treatment with the functional inhibiting anti-TLR2 antibody T2.5 (5 μ g/2 μ L) 15 minutes before the ICV injection of α -synOs (1 μ M/7.5 μ L).

7.2 Results

7.2.1 Alpha-synO-mediated memory impairment is associated with hippocampal neuroinflammation

To determine whether α -synOs triggered the activation of glial cells in our acute mouse model, C57BL/6 naïve mice were ICV injected with α -synOs (1 μ N/7.5 μ L) and their gliosis was investigated at 2, 4, 8 and 24 hours post-injection. By immunofluorescence, we found that both astrocyte (GFAP) and microglial (IBA1) activation in the hippocampus was mainly detectable 4 hours after the α -synOs injection (Figure 27), whereas at 8 and at 24 hours post-treatment glial cells returned close to a resting state similar to that of Veh-treated mice.

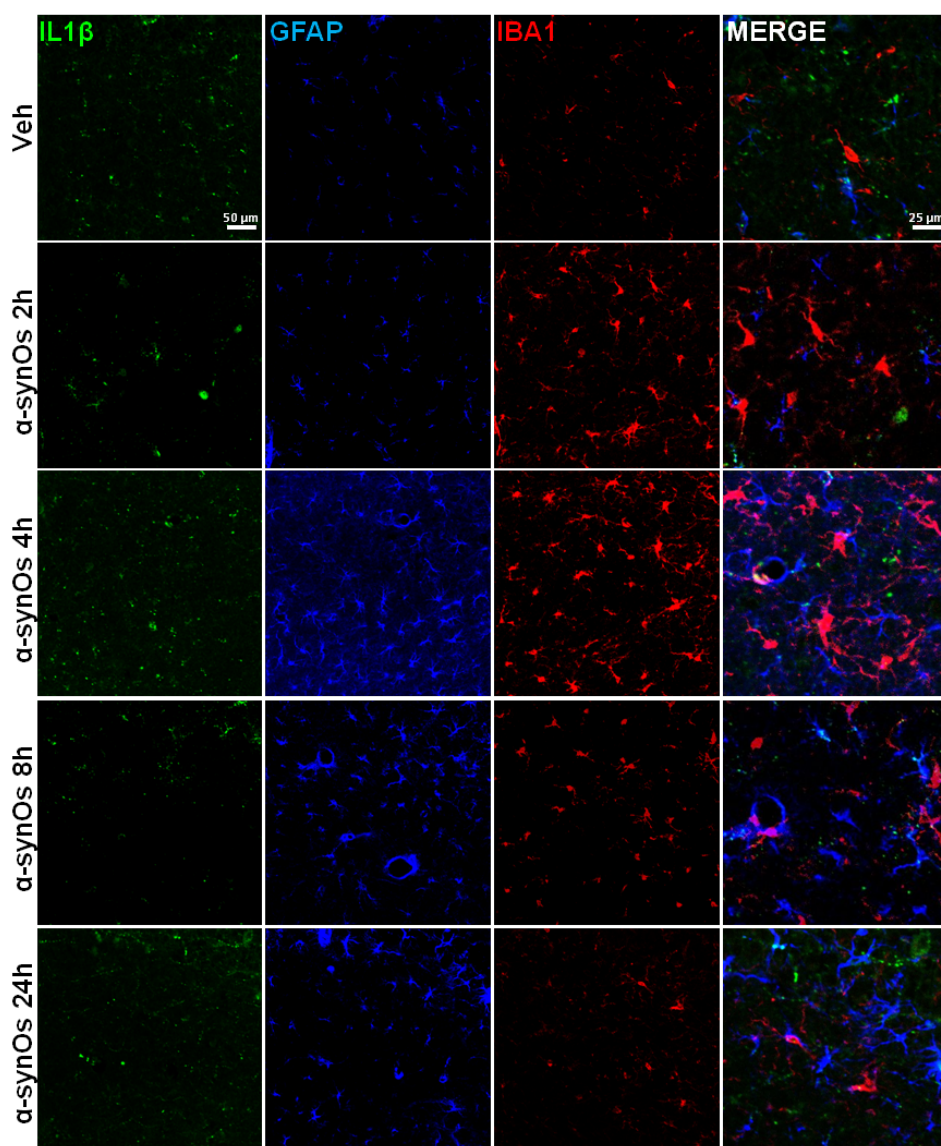


Figure 27. A single α -synO injection induces transient glial cell activation in hippocampus. Representative immunofluorescence images showing hippocampal slices stained for astrocytes (blue), microglia (red) and IL-1 β (green). While a significant glial cell activation occurs 4 hours after α -synOs injection compared to mice receiving Veh, no difference in the expression of IL-1 β is detectable.

We recently reported that a single injection of A β Os in a similar acute mouse model impaired memory in close association with gliosis and increased expression of the pro-inflammatory cytokine IL-1 β which peaks at 8 hours post-injection, and mediated A β O-induced memory damage (Balducci *et al.*, 2017). Based on this evidence, we investigated whether a similar outcome also occurred in α -synO-treated mice. In contrast to A β Os, we found that α -synOs treatment did not lead to increased IL-1 β levels (Figure 27 and 28). Thus, we hypothesised that different pathways may at play in mediating the neuroinflammatory response triggered by α -synOs or A β Os. Of note, the different time point chosen for our investigation in α -synO- or A β O-treated mice was based on our previous evidence (Balducci *et al.*, 2017) showing that in A β O-treated mice gliosis peaked at 8 hours after the ICV injection. Thus, we have investigated IL-1 β expression at the time points corresponding to the maximal glial activation (4 hours after α -synOs and 8 hours after A β Os ICV injection).

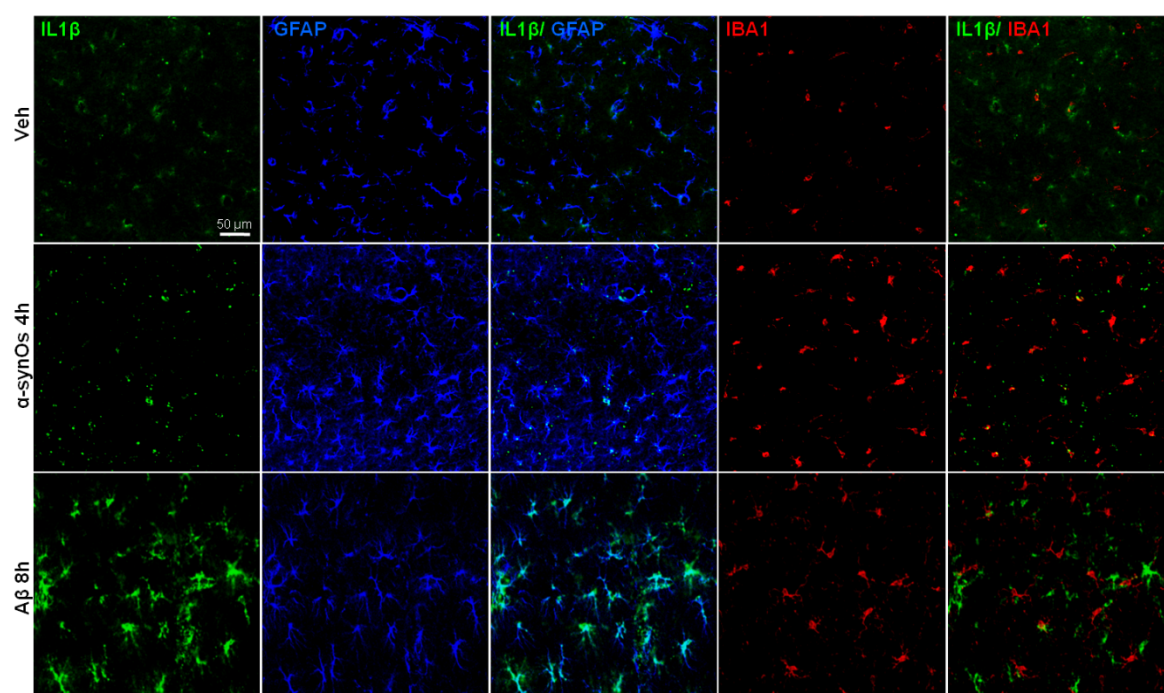


Figure 28. A single α -synO injection does not increase the expression of the pro-inflammatory cytokine IL-1 β . Representative images comparing hippocampal astrogliosis (blue), microgliosis (red) and IL-1 β (green) expression after ICV injection of Veh, α -synOs (4 hours post-injection; top line) and A β Os (8 hours post-injection; bottom line). Of note, the two time points were chosen based on the maximal glial activation triggered by α -synOs or A β Os in our acute mouse models.

7.2.2 Neuroinflammation is a crucial mechanism involved in α -synO-mediated memory damage

As we demonstrated that α -synOs lead to activation of astrocytes and microglial cells, we checked whether neuroinflammation is implicated in the α -synO-induced recognition memory impairment in our model. To address such a question, we tested whether two NSAIDs (Indo and IBF) could prevent α -synO-induced memory damage. 10mg/Kg Indo, which was previously found to be active in abolishing A β O-induced memory impairment (Balducci *et al.*, 2017), was IP administered 30 minutes before the ICV injection of α -synOs. We found that Indo pre-treatment significantly abolished the memory impairment caused by α -synOs (Figure 29). In fact, while mice injected with Veh+ α -synOs had a significantly lower DI (mean \pm SEM) than Veh+Veh-treated animals (0.005 ± 0.06 and 0.26 ± 0.05 respectively; $**p<0.01$, Two-way ANOVA followed by Tukey's test), mice treated with Indo+ α -synOs had a DI comparable to Veh+Veh-treated mice (0.30 ± 0.04), and higher than mice receiving Veh+ α -synOs (0.005 ± 0.06 ; $**p<0.01$, Two-way ANOVA followed by Tukey's test). Thus, these data support the hypothesis that α -synOs trigger a neuroinflammatory response which in turn contributes to the induction of memory impairment.

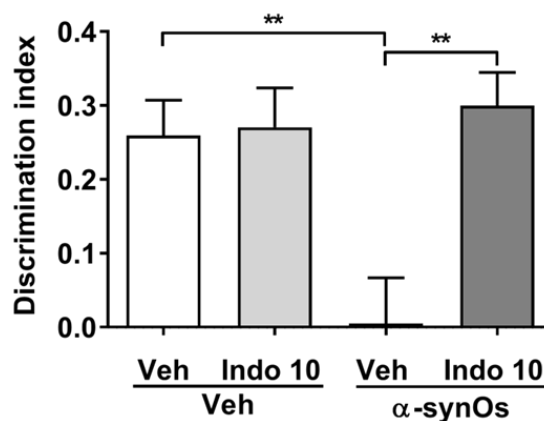


Figure 29. Indo pre-treatment counteracts α -synO-induced memory impairment. Effect of 10mg/Kg Indo pre- α -synOs on memory. Two-way ANOVA found a significant effect of α -synOs ($F_{1,65}=4.4$, $P=0.04$), a significant effect of *Indo* ($F_{1,65}=8.0$, $P=0.0061$) and a significant interaction *Indo* \times α -synOs ($F_{1,65}=6.9$, $P=0.01$). Mean \pm SEM of the DI of mice tested in the NORT (Veh+Veh $n=20$; Indo 10mg/Kg+Veh $n=15$; Veh+ α -synOs $n=19$ and Indo 10mg/Kg+ α -synOs $n=15$; ** $p<0.01$, Tukey's test).

To confirm that the amelioration of mouse memory performances triggered by Indo pre-treatment was due to the lack of α -synO-mediated neuroinflammation, we histologically evaluated the extent of hippocampal gliosis 4 hours post the ICV injection. As described above, this time point was selected based on the evidence that 4 hours post α -synO-treatment corresponded to the time point with the peak of glial cell activation in our model. As shown in figure 30A, we found that Indo pre-treatment led to a reduction of both IBA1- and GFAP-immunoreactivity compared to Veh+ α -synO-treated mice, which was also confirmed by the quantitative analysis of the percentage of IBA1- and GFAP-immunopositive area (Figure 30B and C).

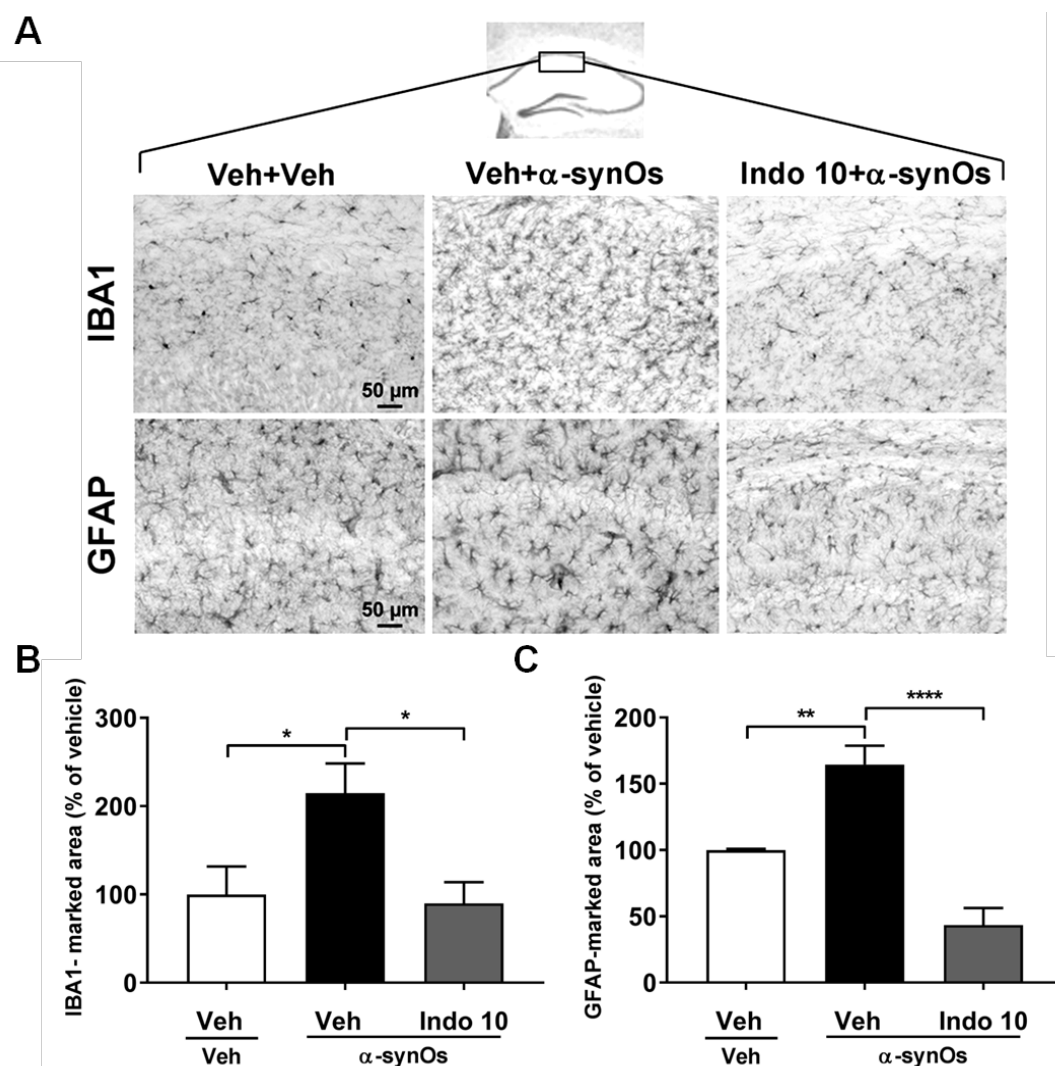


Figure 30. Glial cell activation induced by α -synOs is hampered by Indo pre-treatment. (A) Representative staining for IBA1 (top) and GFAP (bottom) in hippocampal CA1 region of Veh+Veh (n=4), Veh+ α -synOs (n=4) and Indo 10mg/Kg+ α -synOs (n=4) 4 hours post ICV injection. Histograms are the quantitative analysis of the percentage (mean \pm SEM) of marked area for IBA1 (B) and GFAP (C); (*p<0.05, **p<0.01 and ****p<0.001 compared to Veh+ α -synOs; Bonferroni's test).

To further ascertain that the glial response is involved in the detrimental action of oligomers on memory, we pre-treated C57BL/6 mice with a second NSAID, IBF at 5, 10 and 50 mg/Kg (IP 30 minutes before α -synOs). As shown in figure 31, we demonstrated that IBF counteracted the α -synO-mediated memory deficiency. In fact, mice receiving IBF at the dose of 50 mg/Kg and α -synOs (IBF 50+ α -synOs) performed well in the NORT, having a significantly higher DI (mean \pm SEM) than those treated with Veh+ α -synOs (0.24 \pm 0.04 and

-0.06±0.07 respectively; * $p<0.05$, One-way ANOVA followed by Tukey's test), and comparable to that of Veh+Veh treated animals (0.27±0.03). In contrast, IBF at the lower doses of 5 and 10 mg/Kg were ineffective.

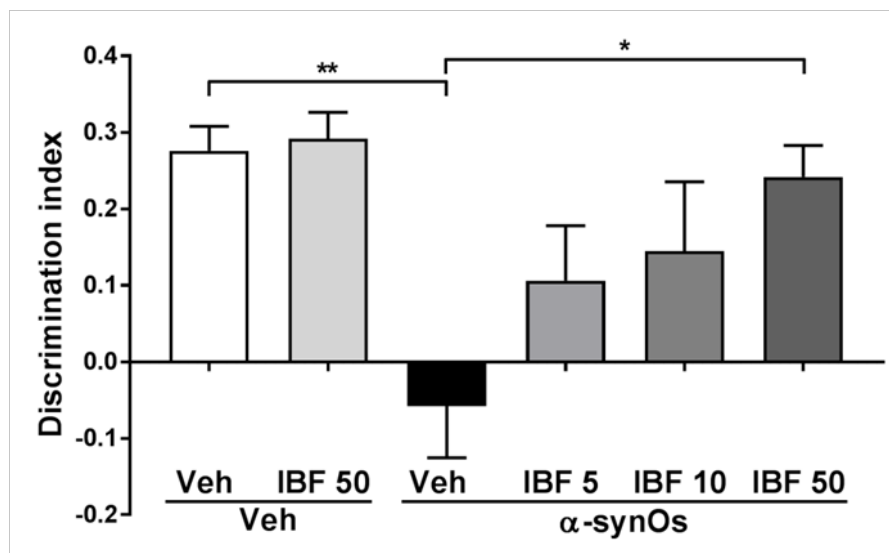


Figure 31. IBF pre-treatment counteracts α -synO-induced memory impairment. Effect of 5,10 and 50mg/Kg IBF pre- α -synOs on mouse memory performances. One-way ANOVA found a significant effect of *treatment* ($F_{5,37}=6.095$, $P=0.0003$) due to the significant impairment induced by α -synOs and the ability of IBF at the dose of 50mg/Kg to abolish it. Histograms are the DI (mean±SEM) of mice tested in the NORT (Veh+Veh $n=10$; IBF 50mg/Kg+Veh $n=6$; Veh+ α -synOs $n=10$; IBF 5mg/Kg+ α -synOs $n=5$; IBF 10mg/Kg+ α -synOs $n=6$ and IBF 50mg/Kg + α -synOs $n=6$; ** $p<0.01$ and * $p<0.05$, Tukey's test).

To pinpoint whether the IBF effect on memory was linked to the blocking of α -synO-mediated glial cell activation, we evaluated the IBF ability in preventing α -synO-mediated hippocampal gliosis 4 hours post ICV injection. As shown in figure 32A, IBF at the active dose of 50 mg/Kg induced a significant reduction of both IBA1- and GFAP-immunoreactivity compared to Veh+ α -synOs-treated mice. Qualitative data were then confirmed by quantitative analysis of the percentage of IBA1- and GFAP-marked area (Figure 32B and C).

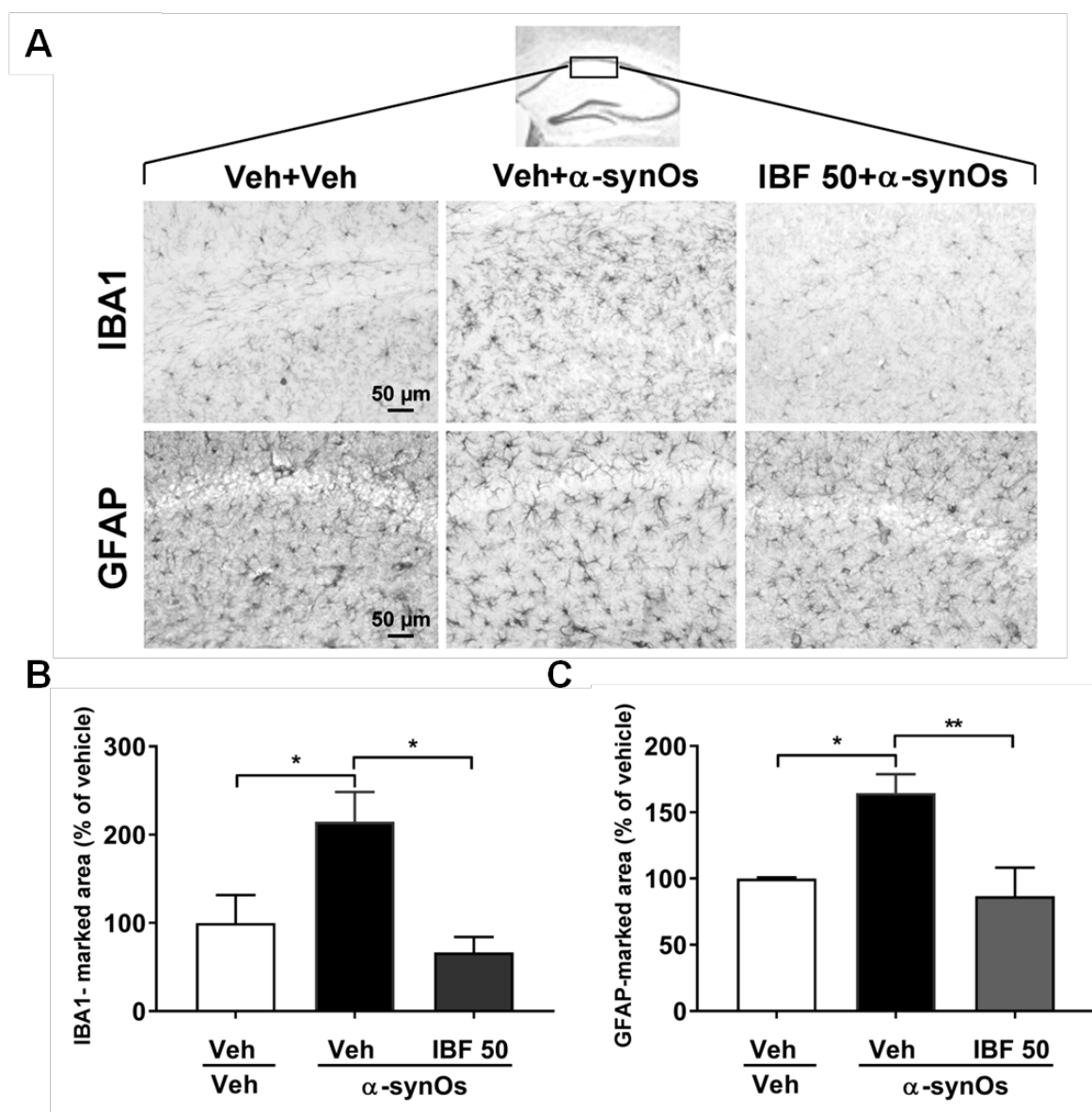


Figure 32. Glial cell activation induced by α -synOs is hampered by IBF pre-treatment. (A) Representative staining for IBA1 (top) and GFAP (bottom) in hippocampal CA1 region of Veh+Veh (n=4), Veh+ α -synOs (n=4) and IBF 50mg/Kg+ α -synOs 4 hours after the ICV injection (n=4). Histograms are the quantitative analysis of the percentage (mean \pm SEM) of marked area for IBA1 (B) and GFAP (C); (*p< 0.05 and **p<0.01 compared to Veh+ α -synOs; Bonferroni's test).

Thus, we herein demonstrate that α -synOs triggered the activation of both astroglial and microglial cells. Moreover, our data show that neuroinflammation is a mechanism involved in mediating the detrimental effects of oligomers on memory. In fact, two different NSAIDs restore mice memory performances suppressing the α -synO-induced neuroinflammatory response.

7.2.3 Alpha-synOs lead to gliosis in the hippocampus whereas monomers and fibrils are ineffective

Our findings indicate that the detrimental effect of oligomeric α -syn on memory establishment is tightly associated to microglial and astroglial cell activation. Hence, we addressed whether monomeric or fibrillar α -syn, which did not affect cognitive performance (described in section 6.2.1), were also ineffective in triggering hippocampal gliosis.

To this aim, we ICV injected C57BL/6 naïve mice with α -syn solutions enriched in monomers, oligomers or fibrils, and both hippocampal IBA1- and GFAP-immunoreactivity were investigated 4 hours post-injection. Consistent with the results reported above, the ICV injection of α -synOs led to a significant increase in both GFAP- and IBA1-marked area compared to Veh-treated animals (Figure 32A-C). In contrast, we found that monomeric and fibrillar α -syn were ineffective (Figure 33A). In fact, our quantitative analysis of both GFAP- and IBA1-marked area confirmed the absence of glial cell activation in mice receiving either monomers or fibrils compared to Veh-treated animals (Figure 33B and C).

Therefore, we herein demonstrate that the neuroinflammatory response is specifically caused by α -synOs. Intriguingly, monomers and fibrils, which do not affect memory establishment are also ineffective in triggering either astrogliosis or microgliosis, strongly supporting the hypothesis that neuroinflammation is a crucial mechanism involved in mediating the harmful effects of oligomers.

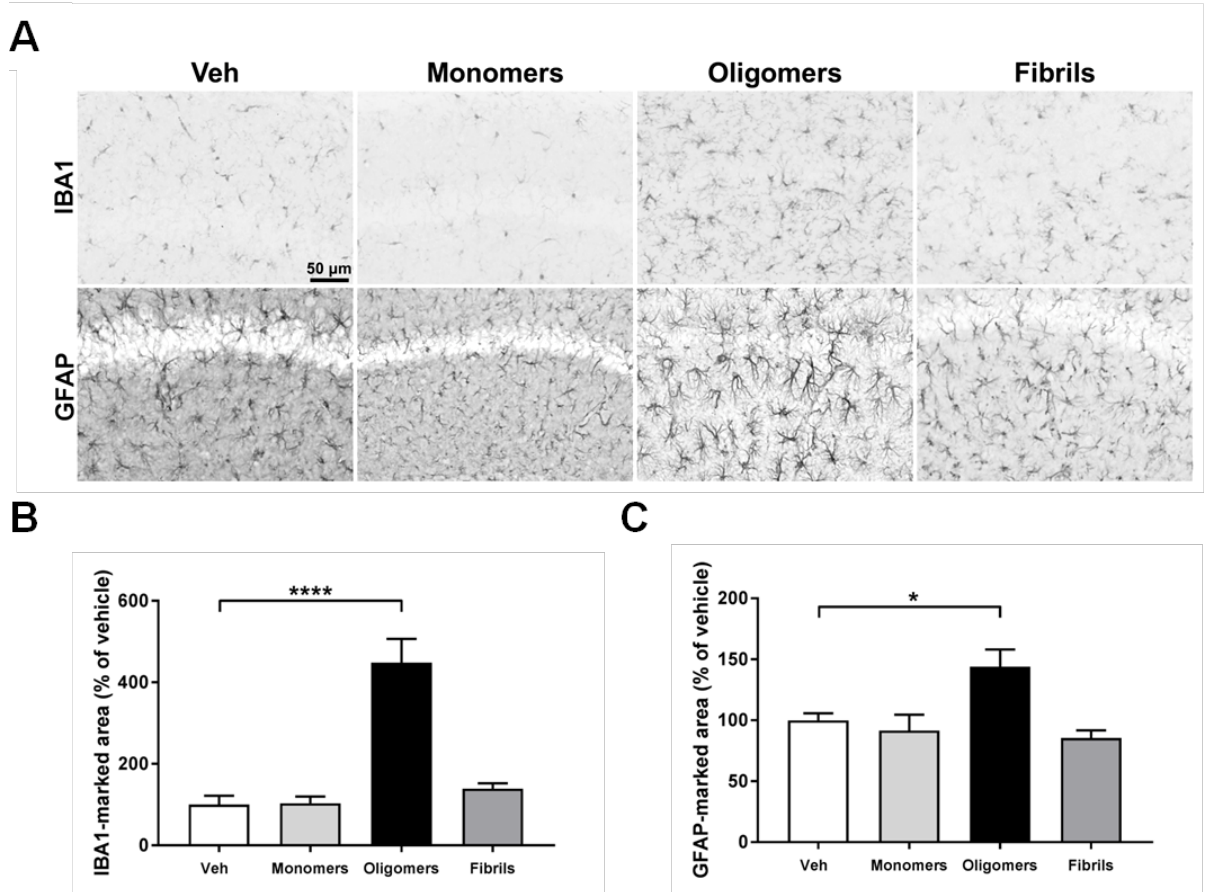


Figure 33. Hippocampal gliosis is specifically induced by α -synOs whereas monomers and fibrils are ineffective. (A) Representative images showing hippocampal slices immunostained for IBA1 (top) or GFAP (bottom) of mice receiving Veh, monomeric, oligomeric or fibrillar α -syn 4 hours post-treatment (4/group). (B, C) Histograms are the quantitative analysis of the marked area percentage (mean \pm SEM) of Veh-, monomer-, α -synO- and fibril-treated mice for either the microglial marker IBA1 (B) or the astroglial marker GFAP (C). One-way ANOVA found a significant effect of *treatment* for both IBA1 ($F_{3,12} = 25.89$, $P < 0.0001$) and GFAP ($F_{3,12} = 6.37$, $P < 0.01$); (*** $p < 0.0001$, * $p < 0.05$ compared to Veh; Bonferroni's test).

7.2.4 Alpha-synO-mediated memory impairment is TLR2-dependent

To get more insight into the immune mechanism underlying the α -synO-mediated memory impairment we focused on TLR4 and TLR2.

Based on the evidence collected through our AD acute mouse model, which indicated that both A β O-mediated memory impairment and neuroinflammation are TLR4 dependent (Balducci *et al.*, 2017), we investigated whether TLR4 could also be responsible for the harmful actions of α -synOs. To this end, α -synOs were ICV injected in TLR4^{0/0} and

subsequently their memory performance was tested in the NORT. As shown in figure 34, we found that α -synOs retained their detrimental action on memory in TLR4^{0/0} mice as well as in WT mice. In fact, TLR4^{0/0} mice receiving α -synOs displayed a significantly lower DI (mean \pm SEM) than TLR4^{+/+} mice injected with Veh (0.01 \pm 0.04 and 0.28 \pm 0.05 respectively; **p<0.01, Two-way ANOVA followed by Tukey's test).

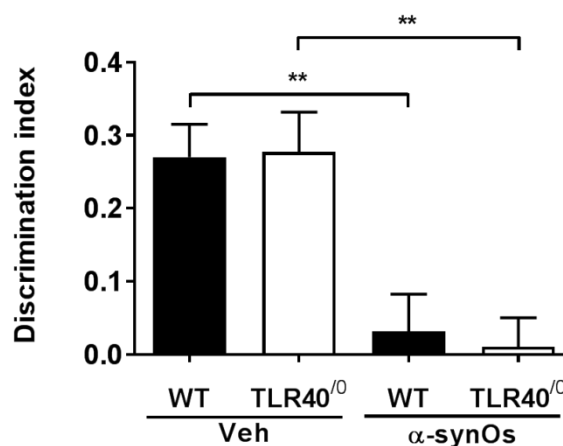


Figure 34. Alpha-synO-triggered memory damage is TLR4-independent. Histograms are the DI (mean \pm SEM) of either C57BL/6 naïve mice or TLR4^{0/0} mice ICV injected with Veh or α -synOs (C57+Veh n=11, C57+ α -synOs n=7, TLR4^{0/0}+Veh n=7 and TLR4^{0/0}+ α -synOs n=7). Two-way ANOVA found a significant effect of α -synOs ($F_{1,28}=23.3$) but a not significant effect of *genotype* ($F_{1,28}=0.02$, $P=0.89$), and a not significant interaction *genotype* \times α -synOs ($F_{1,28}=0.07$, $P=0.79$; *p<0.05 and **p<0.01, Tukey's test).

Accordingly, as described in chapter III, Kim and colleagues reported that α -synO non-cell-autonomous mechanisms, such as activation of glial cells, might occur via TLR2 and are independent of TLR4 (Kim *et al.*, 2013; Kim *et al.*, 2016). To address whether α -synOs exert their deleterious action on memory in a TLR2-dependent manner, we pre-treated C57BL/6 naïve mice with the functional blocking antibody T2.5 at the dose of 2.5 μ g/2 μ L ICV 15 minutes before the injection of Veh or oligomers. In stark contrast with the data we obtained in TLR4^{0/0} mice, the T2.5 pre-treatment completely abolished the α -synO-mediated memory deficiency (Figure 35). In fact, T2.5+ α -synO-treated mice performed well in the NORT, showing a DI (mean \pm SEM) significantly higher than those receiving Veh+ α -synOs

(0.38 ± 0.05 and 0.01 ± 0.05 respectively: **** $p < 0.0001$, Two-way ANOVA followed by Tukey's test).

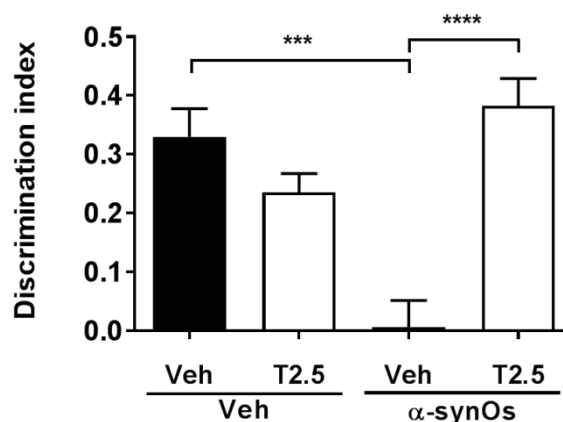


Figure 35. Alpha-synO-triggered memory damage is TLR2-dependent. Histograms are the DI (mean±SEM) of mice ICV injected with Veh+Veh, T2.5+Veh, Veh+α-synOs and T2.5+α-synOs (n=7/group). Two-way ANOVA found a significant interaction $T2.5 \times \alpha\text{-synOs}$ ($F_{1,24}=29.24$, $P=0.0001$; ** $p < 0.0001$ and **** $p < 0.00001$, Tukey's test).

Collectively, our data are consistent with the evidence provided by Kim and colleagues, and indicate that α-synOs impair memory establishment through pathways other than those involved in mediating AβO deleterious effects. In particular, our findings indicate that α-synOs act in a TLR2-dependent manner and independently of TLR4.

7.3 Discussion

Originally introduced in the context of AD, the oligomeric hypothesis has been recently applied to PD and PD-related disorders. Consistently, oligomeric aggregates of Aβ as well as α-syn are identified as the main harmful species underlying the pathogenesis of AD and PD, respectively (Scott *et al.*, 2010; Winner *et al.*, 2011; Mucke and Selkoe, 2012; Balducci and Forloni, 2014; Forloni *et al.*, 2016; Ono, 2017). In fact, while both AβOs and α-synOs have been reported to affect synaptic plasticity and to impair memory, their monomers and fibrils are ineffective (Balducci *et al.*, 2010; Diogenes *et al.*, 2012; Martin *et al.*, 2012; La Vitola *et al.*, 2018).

As previously described, in pathological and stress conditions neurons increase the amount of α -synOs being secreted in their milieu. Thus, extracellular α -synOs may contribute to the pathogenesis of PD and PD-related disorders by exerting their detrimental action in a non-cell autonomous fashion which involves neurons, astrocytes and microglial cells. In this regard, many groups started focusing their efforts on the non-cell autonomous actions of α -synOs. Their data demonstrate that oligomeric α -syn triggers the activation of glial cells, which in turn produce and release several pro-inflammatory mediators leading to neuroinflammatory damage of neurons (Fellner *et al.*, 2013; Kim *et al.*, 2013; Ramikko *et al.*, 2015; Kim *et al.*, 2016). The role of neuroinflammation in mediating memory damage is strongly supported by evidence from animal models of AD. In an acute mouse model of AD, A β Os impair memory performance through glial activation and increase of pro-inflammatory cytokines expression (Balducci *et al.*, 2017). Intriguingly, the block of neuroinflammation resulted in the rescue of memory functions both in an acute and in the APP/PS1 Tg mouse model of AD (Balducci *et al.*, 2017; Balducci *et al.*, 2018). Since α -synOs have been detected in the extracellular fluids, researchers have speculated that α -synOs like A β Os may exert their own detrimental activities through indirect non-cell-autonomous mechanisms which include glial cell activation. Thus, neuroinflammation might represent a process underlying cognitive decline in the context of α -synucleinopathies. Despite initially considered as a consequence of the neurodegenerative process which takes place in PD and its related disorders, neuroinflammation is now emerging as a mechanism fostering α -synO deleterious activities.

Astrocytes as well as microglial cells have vital roles at the neuronal level, and they are crucial players in the fine tuning of neuronal activity and synaptic plasticity (Bacci *et al.*, 1999; Blank and Prinz, 2013; Hertz and Chen, 2016). Therefore, perturbation of their function/state might lead to neuronal damage, dysfunction and ultimately death (Morris *et al.*, 2013; Blank and Prinz, 2013).

To decipher the role of the neuroinflammatory response in mediating the memory damage triggered by α -synOs, we investigated whether the memory deficit mediated by α -synOs in our acute mouse model was associated with glial cell activation. Our results indicated that, a single ICV injection of α -synOs triggered a transient activation of glial cells in the hippocampus. In fact, we found that 4 hours after treatment glial cells displayed an increase in cell density and body enlargement compared to mice receiving Veh. In contrast to A β Os, the expression of IL-1 β after α -synOs did not change. This difference is intriguing and highly significant. In fact, although protein-misfolding neurodegenerative disorders share common neuropathological features, they may differ in the action mechanism of oligomers derived from their own related causative protein.

To ascertain whether α -synO-mediated neuroinflammation was involved in the memory damage caused by α -synOs in the acute mouse model we developed, we pre-treated mice with either Indo or IBF, two NSAIDs able to cross the BBB and to inhibit both cyclooxygenase I (COX1) and cyclooxygenase II (COX2). We found that Indo and IBF both ameliorated the memory performances of α -synO-treated mice. Moreover, the behavioural effect of Indo and IBF pre-treatment correlated with a drastic protection against α -synO-mediated hippocampal gliosis. Thus, our findings highlight the implication of glial cell activation in the α -synO-mediated memory impairment, and they are consistent with previous data reported by Drouin-Ouellet and co-workers. In fact, they demonstrate that the treatment of a Tg PD mouse model with a COX-2 inhibiting anti-inflammatory drug abrogates neuroinflammation and restores motor behaviour performances (Drouin-Ouellet *et al.*, 2015).

The initiation of the inflammatory response generally starts after the recognition of DAMPs or PAMPs by PRRs such as TLRs. TLRs are first line players involved in the innate immune response and are able to detect both exogenous and endogenous signals including misfolded and aggregated α -syn. TLRs have been widely investigated in the context of PD, and despite

their contribution in α -synucleinopathies is controversial and debated, evidence for TLR2 or TLR4 engagement in such pathologies raises from several observations (Letiembre *et al.*, 2009; Béraud *et al.*, 2011; Fellner *et al.*, 2013; Kim *et al.*, 2013; Drouin-Ouellet *et al.*, 2015; Rannikko *et al.*, 2015; Kim *et al.*, 2016; Kouli *et al.*, 2019).

To shed light on this aspect and to reveal potential mechanisms of action of α -synOs, we investigated whether TLR4 mediates memory loss. Our findings demonstrate that memory impairment caused by α -synOs is TLR4-independent. Indeed, α -synOs ICV injected either in C57BL/6 naïve mice or in TLR4^{0/0} mice equally impaired their memory. On the other hand, we showed that the functional block of TLR2 completely preserved memory loss in mice ICV treated with oligomers.

To assess the role of TLR4 in mediating α -synO detrimental effect on memory we used TLR4^{0/0} mice, whereas to investigate the involvement of TLR2 a pharmacological approach with a specific inhibitor was carried out. The reason why we opted for such a tool is that a reliable model of TLR2^{0/0} is available solely on the 129 mouse background, which are not suitable for cognitive behavioural studies.

One may speculate that the effect of our TLR2 functional blocker (T2.5) could result from a direct interaction of T2.5 with α -synOs, which might prevent oligomers from exerting their detrimental actions. Although this possibility has not been addressed in this thesis, some evidence exists that can rule out this hypothesis: 1) T2.5 has been described as a high specific blocker for TLR2, and 2) several studies exploiting the TLR2 blocker did not report any direct binding with α -synOs, but only indirect effects such as block of α -synO neuron-to-neuron transmission or inhibition of the inflammatory response after α -synOs exposure (Kim *et al.*, 2013; Kim *et al.*, 2016; Kim *et al.*, 2017).

Thus, our data clearly indicate that α -synOs exert their harmful effects in a TLR2-dependent manner and they support TLR2 as a potential candidate for therapeutic approaches.

Consistently, Kim and colleagues recently report that the immunotherapy against the TLR2 in a Tg PD mouse model leads to neuroprotective effects (Kim *et al.*, 2018).

Collectively the results here described pinpoint the modulation/inhibition of inflammatory mediators (COX1, COX2 and TLR2) as valuable strategies to counteract α -synO-mediated deleterious effects. However, as COX1, COX2 and TLR2 are also expressed by neurons, we cannot rule out a direct action of α -synOs on neurons resulting in subsequent glial activation. Of note, recently, Dzamko and co-workers showed that TLR2 is up-regulated in both microglia and neurons within the brain of PD patients and that the neuronal stimulation through TLR2 agonists leads to a significant increase in the expression of pro-inflammatory mediators by neurons themselves (Dzamko *et al.*, 2017). Thus, neurons appear to play an active role in the neuroinflammatory process in concert with glial cells. A synergistic action of α -synOs both on neurons and glial cells is more likely plausible than an effect of extracellular oligomers exclusively on neurons.

In conclusion, our findings strongly support the involvement of the neuroinflammatory response in mediating detrimental effects of α -synOs and further depict neuroinflammation as a crucial player in the pathogenesis of α -synucleinopathies, and not merely as a secondary event triggered by the underlying neurodegeneration. Moreover, the acute mouse model we developed appears as a valid tool to specifically investigate the detrimental activities of α -synOs and to investigate their action mechanisms.

Results

Chapter VIII

***Alpha-synO harmful actions are not dependent on
the cellular Prion protein***

The data presented in this chapter are reported in the manuscript entitled “Cellular prion protein neither binds to alpha-synuclein oligomers nor mediates their detrimental effects”. Brain. 2019 Feb 1;142(2):249-254. doi: 10.1093/brain/awy318.

8.1 Aim of the study and experimental approaches

To uncover the mechanisms underlying the detrimental effects of α -synOs, several biological fields have been widely explored. The most common scenarios taken into account to explain how oligomers induce cellular dysfunction and eventually death include: I) alterations in membrane permeability (Danzer *et al.*, 2007; Tsigelny *et al.*, 2012;); II) neuroinflammation (Fellner *et al.*, 2013; Kim *et al.*, 2013; Kim *et al.*, 2016; La Vitola *et al.*, 2018); III) protein-protein interaction (Betzer *et al.*, 2015).

With regard to protein-protein interaction, a recently emerged oligomer-protein interactor is PrP^C, which was initially described as mediator of A β Os detrimental effects and more recently proposed as interactor of α -synOs (Lauren *et al.*, 2009; Ferreira *et al.*, 2017).

Based on these findings, we aimed to further investigate the α -synO-PrP^C interaction using direct approaches. *In vitro*, we compared the toxicity of α -synOs in either PrP^{+/+} or PrP^{0/0} primary hippocampal neurons. *In vivo*, attempting to elucidate the role of the PrP^C in mediating the α -synO-induced memory deficit, we exploited the acute mouse model described in chapters VI and VII. Specifically, both PrP^{+/+} and PrP^{0/0} mice were ICV injected with Veh or α -synOs (1 μ M/7.5 μ L) 2 hours before the familiarisation/sample phase of the NORT. Thus, memory performance of each mouse was assed 24 hours later (Figure 36).

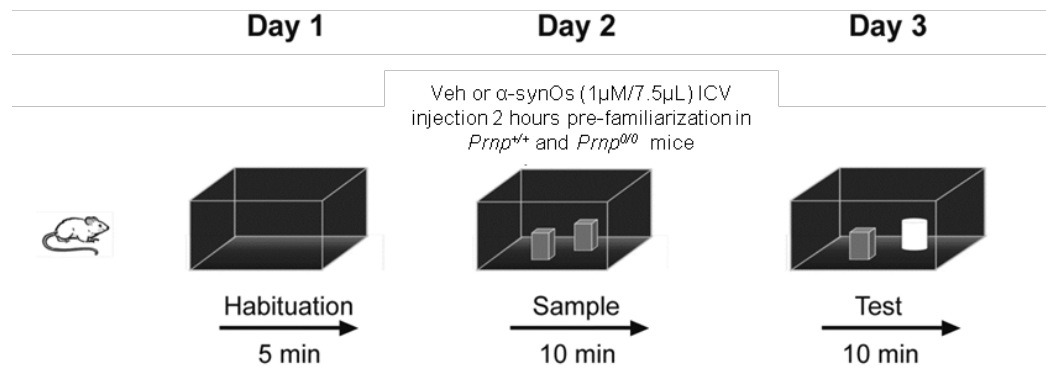


Figure 36. Schematic representation of treatment schedule for the NORT experiments. α -synOs (1 μ M/7.5 μ L) in both $Prnp^{+/+}$ and $Prnp^{0/0}$ were ICV injected 2 hours before the familiarisation/sample phase.

We previously reported that the neuroinflammatory response triggered by the ICV injection of α -synOs is crucial in mediating their detrimental activities on memory (chapter VII and La Vitola *et al.*, 2018). Therefore, we further investigated the role of the PrP^C in mediating the hippocampal astroglial and microglial cell activation in either PrP^{+/+} or PrP^{0/0} mice 4 hours after the ICV injected with Veh or α -synOs (1 μ M/7.5 μ L). Of note, assessment of the activation of glial cells has been performed at this time point since it corresponds to the maximal glial activation in our acute model (chapter VII and La Vitola *et al.*, 2018). In addition, at the molecular level, we verified the existence of a direct binding between α -synOs and PrP^C by SPR, using the same approach previously exploited in our laboratory in the biochemical studies on the A β O-PrP^C interaction (Balducci *et al.*, 2010).

8.2 Results

8.2.1 Alpha-synO-mediated detrimental effects are PrP^C-independent

To specifically address the PrP^C involvement in mounting α -synO harmful effects, we took advantage of both *in vitro* and *in vivo* approaches. In particular, to assess whether the PrP^C is required for triggering α -synO-induced toxicity *in vitro*, we measured both *Prnp*^{+/+} and *Prnp*^{0/0} primary hippocampal neuron survival upon 48 hours exposure to α -synOs at 1, 5 and 10 μ M. In order to ascertain the presence of α -synOs, we had previously characterised our preparation at all doses being used in our experiments through AFM and Western Blotting. As shown in figure 37, the results obtained through our AFM analyses proved the presence of oligomeric assemblies with similar diameter ranging from 15 to 40 nm and notably demonstrate the absence of larger aggregates in each condition investigated (Figure 37A-C left and middle panel). In line with the AFM data, a comparable distribution of oligomeric assemblies at 1, 5 and 10 μ M was also demonstrated by our western blotting analyses (Figure 37A-C right panel), which showed the presence of SDS-resistant α -synOs with a molecular weight ranging from 35 to 100 KDa in each preparation.

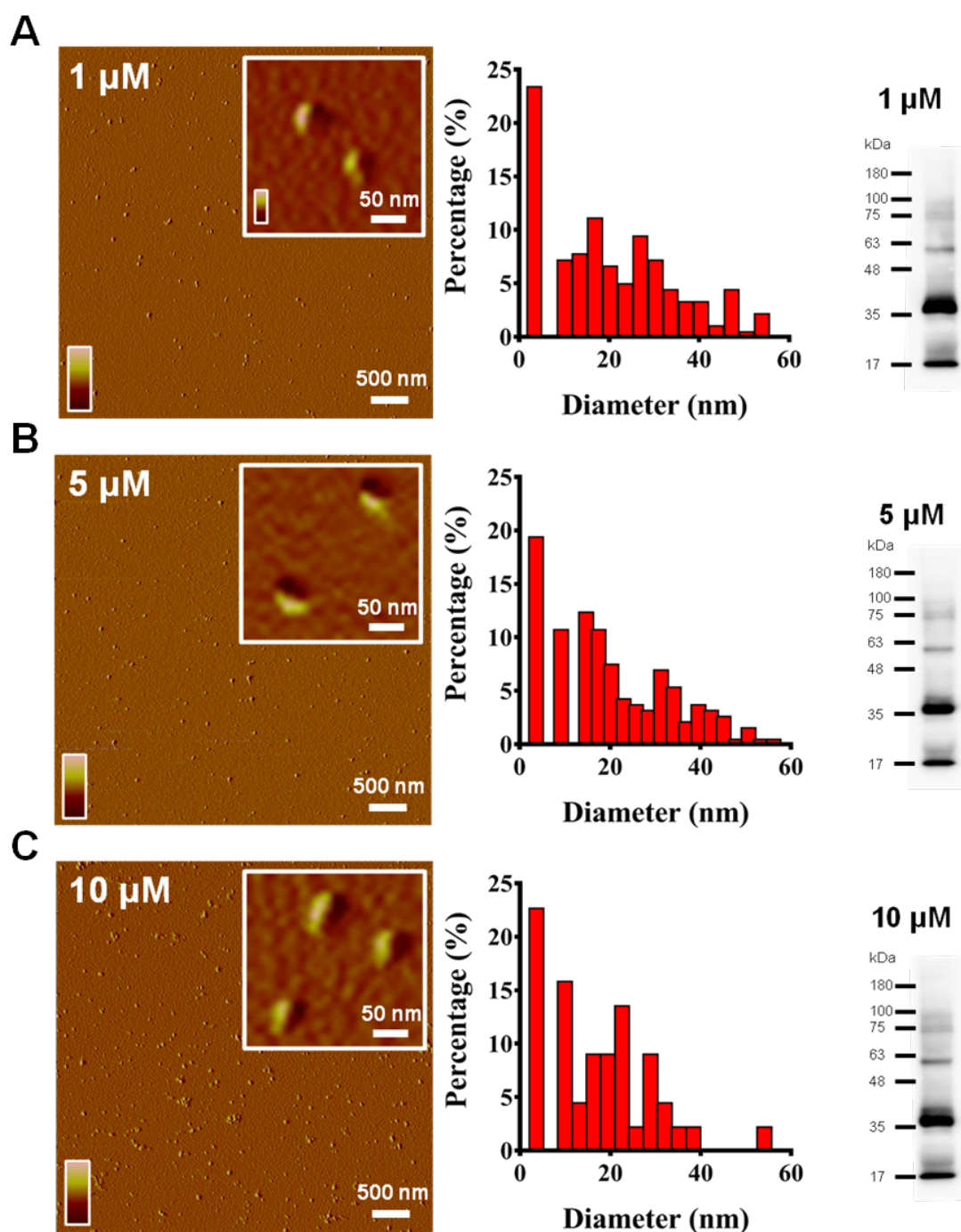


Figure 37. Atomic Force Microscopy and Western Blotting: characterisation of α -synO preparation (1, 5 and 10 μM). Representative tapping mode of AFM images (left panels) as determined by amplitude data of oligomeric samples at 1 (**A**), 5 (**B**) and 10 μM (**C**). The colour scale bars correspond to an amplitude range of -15 to $+15$ mV for the main figures and -12 to $+20$ mV for insets. Histograms (middle panels) show the diameter frequency distribution of the different assemblies obtained for 1 (**A**), 5 (**B**) and 10 μM (**C**). The diameter frequency is similar for the 3 preparations with a distribution between 15 and 40 nm. Western blot analysis (right panels) of α -synOs at 1 (**A**), 5 (**B**) and 10 μM (**C**) showing different SDS-resistant assemblies ranging from 35 to 100 KDa.

In vitro, we found that the PrP^C was not a prerequisite for α -synO detrimental effects. In fact, independently of the genotypes considered (*Prnp*^{0/0} and *Prnp*^{+/+}), the 48 hours exposure of hippocampal neurons to α -synOs (1, 5, and 10 μ M) led to a significant reduction of the neuronal viability measured through the MTT assay (Figure 38), and as proved by the two-way ANOVA showing a significant effect of α -synOs ($F_{3, 46}=24.7$; $P<0.0001$) but a non-significant interaction α -synOs \times genotype ($F_{3, 46}=0.70$; $P=0.56$). Thus, our evidence indicates that α -synO cytotoxicity is not dependent on the PrP^C.

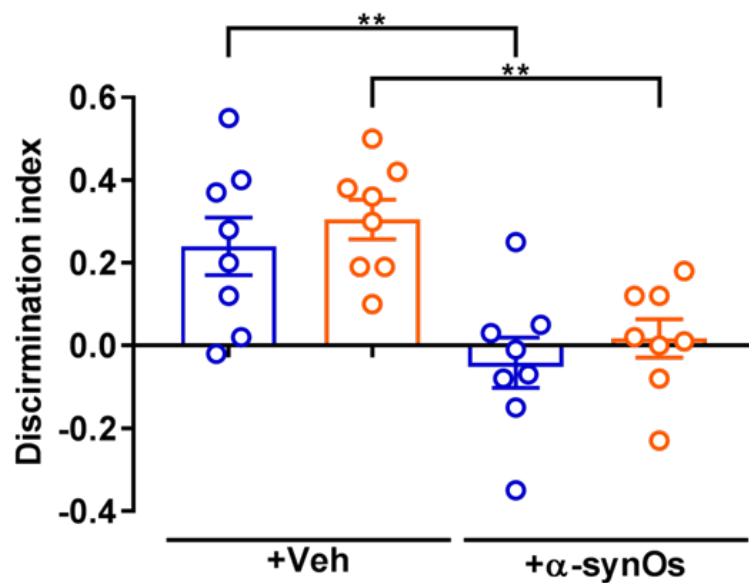


Figure 38. Alpha-synO-mediated cytotoxicity is PrP^C-independent. (A) Scatter plots with bars are the percentage of cell survival in MTT assay 48 hours after treatment with Veh or α -synOs at 1, 5 and 10 μ M (* $p<0.05$ and **** $p<0.0001$ against Veh; Two-way ANOVA followed by Tukey's test).

To further investigate whether α -synO detrimental effects are PrP^C-mediated, we tested the memory performance in both *Prnp*^{0/0} and *Prnp*^{+/+} mice ICV injected with either Veh or α -synOs (1 μ M/7.5 μ L). Thus, this direct and simple approach, which exploited the acute mouse model previously described, allowed us to specifically assess the effects of α -synOs on long-term memory (chapters VI and VII; La Vitola *et al.*, 2018). As shown in figure 39, both *Prnp*^{+/+} and *Prnp*^{0/0} mice displayed a significant memory impairment after ICV treatment with α -synOs compared to mice receiving Veh. In fact, the DI (mean \pm SEM) of both *Prnp*^{+/+}

and *Prnp*^{0/0} receiving α -synOs (-0.04 ± 0.06 and 0.02 ± 0.05 respectively) was significantly lower than those of both *Prnp*^{+/+} and *Prnp*^{0/0} receiving the Veh (0.24 ± 0.07 and 0.31 ± 0.05 respectively; $**p<0.01$, Two-way ANOVA followed by Tukey's test). Our findings suggest that, at the functional level, α -synOs act independently of PrP^C. In fact, the Two-way ANOVA showed a significant effect of α -synOs ($F_{1,28}=24.89$; $P<0.0001$), but a non-significant interaction α -synOs \times genotype ($F_{1,28}=0.003$; $P=0.96$).

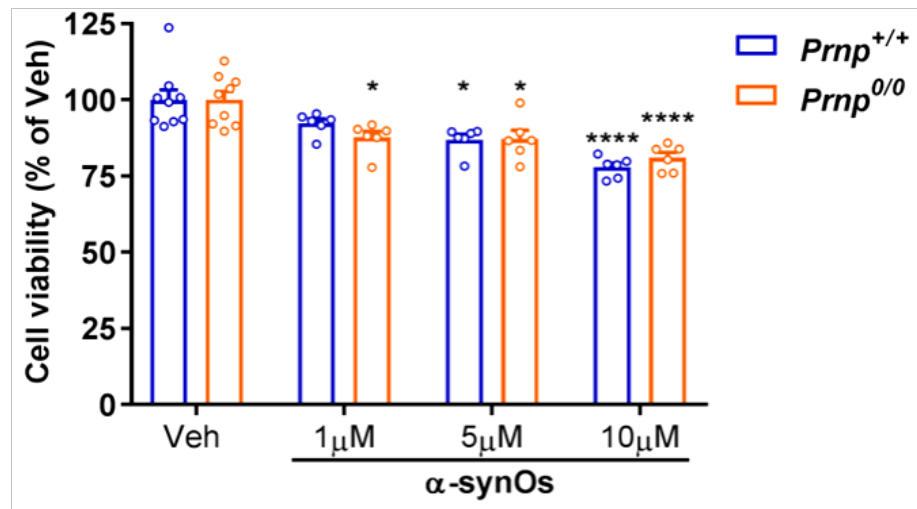


Figure 39. Alpha-synO-mediated detrimental action on memory is PrP^C-independent. Scatter plots with bars are the DI (mean \pm SEM) of *Prnp*^{+/+} and *Prnp*^{0/0} mice treated with Veh or α -synOs ($**P<0.01$, Two-way ANOVA followed by Tukey's test; $n=8$ /group).

Based on our previous findings (chapter VII and La Vitola *et al*, 2018), showing that in our acute mouse model α -synO-mediated memory damage was tightly associated to a transient hippocampal gliosis, we addressed whether a similar outcome could also take place in *Prnp*^{0/0} mice. Through histological analysis of astrocytes (GFAP) and microglia (IBA1), we assessed the extent of glial activation 4 hours after treatment. As shown in figure 40A and B, we found that α -synOs led to an increased expression of GFAP and IBA1 in both *Prnp*^{+/+} and *Prnp*^{0/0} mice when compared to those receiving Veh. Accordingly, as shown in figure 40C and D, our qualitative analyses were also confirmed by quantifications. Specifically, comparing the percentage of hippocampal GFAP- or IBA1-immunopositive area in the

different experimental groups investigated, we discovered that the α -synO-induced gliosis was PrP^C-independent. In fact, the Two-way ANOVA indicated a significant effect of α -synOs (GFAP: $F_{1,16}=24.45$; $P=0.0001$. IBA1: $F_{1,16}=57.31$, $P<0.0001$) but a non-significant interaction α -synOs \times genotype (GFAP: $F_{1,16}=0.041$, $P=0.84$. IBA1: $F_{1,16}=0.50$, $P=0.49$).

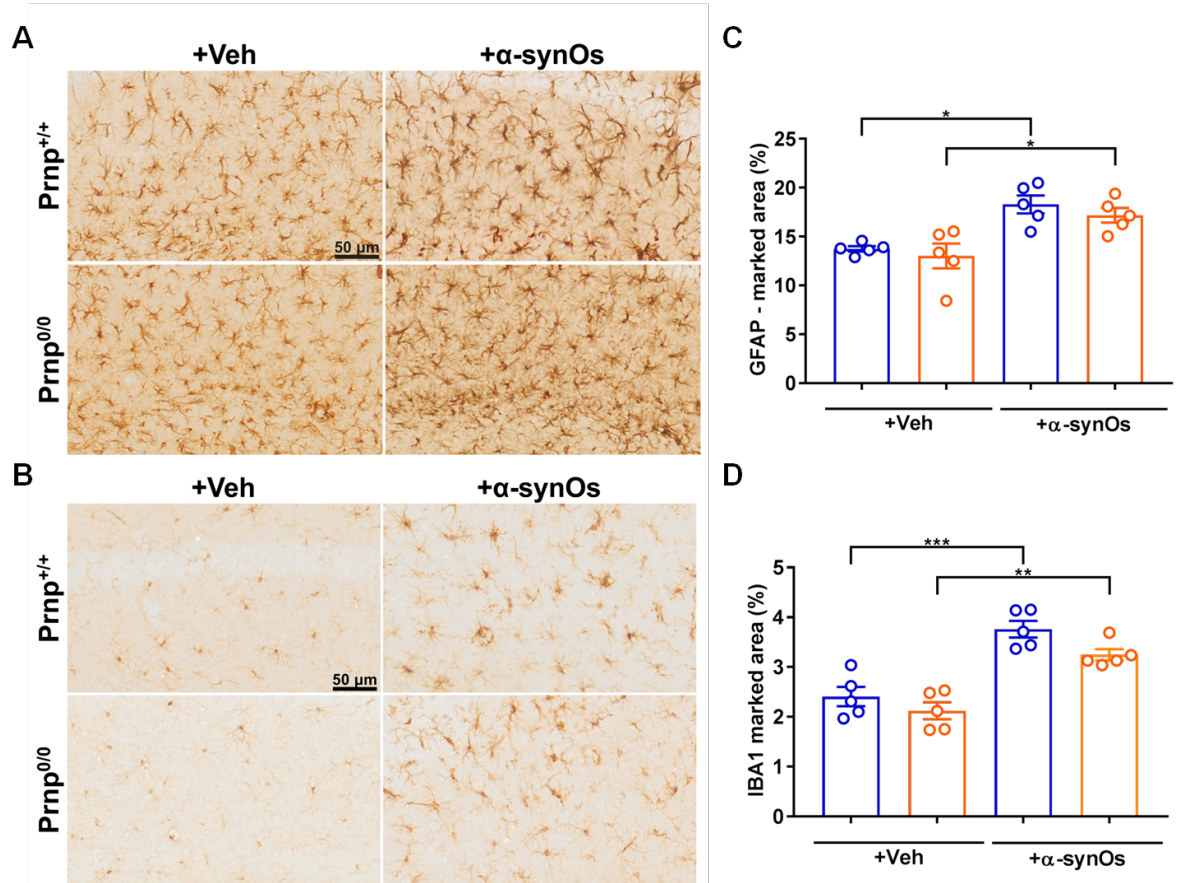


Figure 40. Alpha-synO-induced hippocampal gliosis is PrP^C-independent. (A) Representative images of GFAP-immunostaining in the hippocampus of mice treated with either Veh or α -synOs. (B) Representative images of IBA1-immunostaining in the hippocampus of mice treated with either Veh or α -synOs. (C) Scatter plots with bars of the relative GFAP-marked area quantification in $Prnp^{+/+}$ and $Prnp^{0/0}$ mice treated with Veh or α -synOs (* $p<0.05$. Two-way ANOVA followed by Tukey's test; $n=5$ /group). (D) Scatter plots with bars of the relative IBA1-marked area quantification in $Prnp^{+/+}$ and $Prnp^{0/0}$ mice treated with Veh or α -synOs (** $p<0.01$ and *** $p<0.001$. Two-way ANOVA followed by Tukey's test; $n=5$ /group).

Together, our findings indicate that from a functional standpoint the PrP^C is not mandatory for mediating the detrimental effects of α -synOs at multiple levels, such as neuronal cell toxicity, memory impairment and neuroinflammation.

8.2.2 Alpha-synOs and PrP^C do not directly interact

To assess the PrP^C- α -synO direct binding, we exploited the SPR approach. PrP^C from mouse brain homogenates was captured on SPR sensor chips coated with 3F4 or 94B4 anti-PrP^C antibodies. Of note, we selected 3F4 and 94B4 based on the evidence that the PrP^C residues 93-109 have been identified as crucial for the α -synO interaction (Ferreira *et al.*, 2017), and on the evidence that our two antibodies recognise epitopes lying within regions 106-111 and 186-193 respectively. In a previous paper, we had verified that the captured protein is actually PrP^C, as no capture was detected when flowing brain homogenate obtained from *Prnp*^{0/0} mice (Balducci *et al.*, 2010). Moreover, PrP^C captured by either 94B4 or 3F4 retained its ability to bind 6D11, an anti-PrP^C antibody directed against the same region (93-109) which was described to be involved in α -synO-induced toxic effects (Balducci *et al.*, 2010; Ferreira *et al.*, 2017). Under all these conditions, when α -synOs (1 and 10 μ M) were flowed over captured PrP^C we could not find any α -synO-PrP^C binding (Figure 41A and B). In contrast, 1 μ M A β Os, injected in parallel as positive control, produced the expected binding signal (Figure 41A and B).

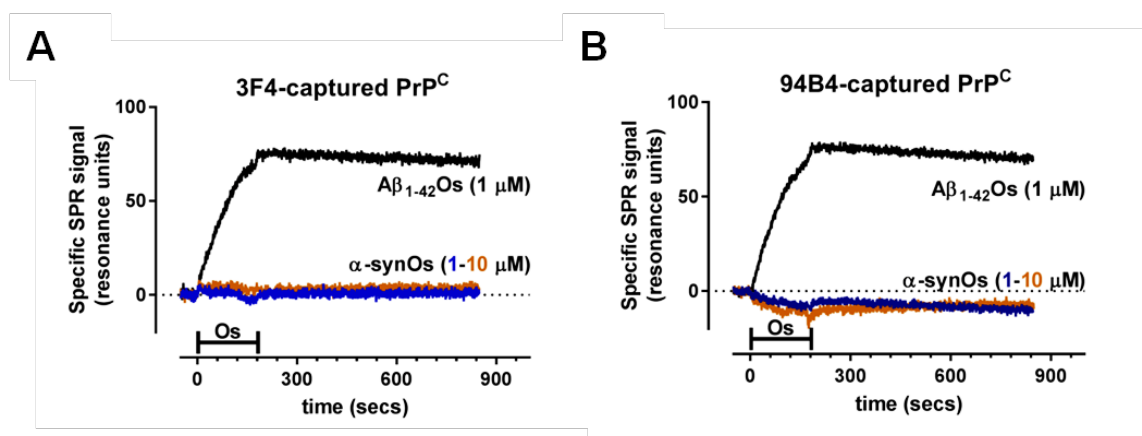


Figure 41. Alpha-synOs do not directly bind to PrP^C. SPR analysis were performed flowing α -synOs (1 or 10 μ M) and A β ₁₋₄₂Os (1 μ M), as positive control, for 3 min over sensor surfaces exposing PrP^C, captured by 3F4 (A) or 94B4 (B) antibodies. Sensorgrams show the time course of the specific PrP^C-dependent SPR binding signal.

Therefore, our results clearly demonstrate the absence of a PrP^C- α -synO direct binding in different experimental conditions and provide molecular evidence of our functional data on the PrP^C-independent action of α -synOs.

8.3 Discussion

Initially considered as mere intermediates forming during the aggregation process of α -syn, the small soluble oligomeric assemblies of α -syn are now widely accepted as crucial players and main pathological drivers in the pathogenesis of PD and PD-related disorders (Winner *et al.*, 2011; Scott *et al.*, 2010; Diogenes *et al.*, 2012; Martin *et al.*, 2012; Ono, 2017; Bengoa-Vergniory *et al.*, 2017). Attempting to elucidate the pathways underlying the α -synO harmful properties, researchers have focused their efforts on both cell-autonomous and non-cell autonomous mechanisms. However, despite a large body of evidence, a clear and univocal mechanism has not been defined yet. Recently, Ferreira and colleagues (Ferreira *et al.*, 2017) have proposed the PrP^C as a novel interactor of α -synOs, which is apparently implicated in mediating their detrimental activities. Particularly, in their study Ferreira and co-workers found that PrP^C interacts with α -synOs, and the PrP^C- α -synO interaction induces the phosphorylation of Fyn kinase and the consequent phosphorylation of NMDAR2B, triggering a signalling cascade that leads to synaptic dysfunction (Ferreira *et al.*, 2017).

To elucidate the role of the PrP^C as mediator for the deleterious activities of α -synOs, in the present study we further investigated this emerging hypothesis through direct *in vitro* and *in vivo* approaches. In order to provide evidence of the oligomeric state in our experimental conditions, we firstly characterised our three α -synO preparations (1, 5 and 10 μ M) through both AFM and Western Blotting. Of note, in our three samples we found a similar distribution of small assemblies without evidence of larger aggregates. Since our protocol for obtaining α -synOs at the three different concentrations is solely based on the incubation

of α -syn monomers at 241 μ M for 48 hours at 37°C with subsequent dilution at 1, 5 and 10 μ M, the similarities in our preparations were expected. Once verified the quality of our oligomers, we performed analyses of the PrP^C- α -synO interaction at multiple levels.

Notably, our SPR analysis did not confirm the existence of a direct PrP^C- α -synO binding in different conditions. Moreover, α -synOs impaired mouse memory and induced gliosis regardless of presence of PrP^C, thus indicating the not mandatory role of the PrP^C as mediator of the α -synO detrimental effects.

Of note, while our findings clearly depict PrP^C as not mandatory for mediating α -synO detrimental effects at functional level, we should take into account some aspects of the SPR approach herein exploited to assess the PrP^C- α -synOs binding. Although, SPR is widely used to detect protein-protein interaction, and provides an efficient tool to investigate direct interactions (Balducci *et al.*, 2012; Shirasaka *et al.*, 2019), bias may occur to a certain extent. In fact, the technique is based on the immobilisation of a protein (PrP^C in our study) on the surface of a sensor chip via binding to a specific antibody. Such an antibody-protein interaction could result in a conformational change of the immobilised protein, that may make the binding site required for the interaction with the analyte (α -synOs in our study) unavailable or masked by steric hindrance. To rule out, at least partially, such a possibility, we ascertained that PrP^C retains its ability to bind A β Os as well as the antibody 6D11, which shares the same binding site of α -synOs on PrP^C (Ferreira *et al.*, 2017). Moreover, as we obtained consistent results using two different antibodies for on-chip immobilisation of PrP^C, it is highly improbable that the same conformational changes take places in both cases. Nonetheless, although our data seem to exclude a PrP^C- α -synOs direct interaction, we cannot completely rule out that our SPR analyses are partially biased by the events described above. On the other hand, it is also relevant to note that the PrP^C- α -synOs interaction described in the work of Ferreira and colleagues, was revealed by an indirect immunoprecipitation

approach. In fact, in the context of a cell lysate several proteins may mediate the PrP^C- α -synOs complex, and therefore the physical protein-protein direct binding cannot be confirmed and demonstrated .

To be noticed that, while our SPR analyses may be biased, our functional studies completely ruled out the role of PrP^C in mediating α -synO-induced neuronal toxicity, cognitive damage and neuroinflammation. Thus, assuming that a PrP^C- α -synOs binding exists, it does not appear to be involved from a neuropathological and behavioural perspective

As demonstrated in chapter VI and recently published, the ICV injection of α -synOs triggers a neuroinflammatory response which drives the α -synO-induced impairment of new memory establishment in C57BL6 naïve mice (La Vitola *et al.*, 2018). Intriguingly, herein our findings depict the occurrence of a neuroinflammatory response caused by α -synOs also in *Prnp*^{0/0} mice. Thus, our data may further support the role of neuroinflammation as potential non-cell autonomous mechanism mediating the harmful activities of α -synOs (La Vitola *et al.*, 2018; La Vitola *et al.*, 2019).

Our data are in contrast with those of Ferreira (Ferreira *et al.*, 2017), and although we have no explanations for this at the state of the art, it could be argued that different α -synO preparations were used. Moreover, a further possible explanation is that other α -synOs conformers might associate with PrP^C, and that both PrP^C-dependent and PrP^C-independent pathways may co-exist in PD as previously reported also for A β Os in the context of AD (Purro *et al.*, 2018).

Results

Chapter IX

***Peripherally induced neuroinflammation influences
 α -synO harmful actions and the PD phenotype in an
acute and transgenic mouse model***

The data presented in this chapter are reported in the recently submitted manuscript entitled “Peripherally-induced neuroinflammation exacerbates alpha-synuclein oligomer toxicity and neuropathology in Parkinson’s disease mouse models”.

9.1 Aim of the study and experimental design

As far, our results highlight neuroinflammation as a mediator for α -synO detrimental activities on memory. However, as previously described in the introduction section, compelling evidence from clinicians and experimental studies depicts neuroinflammation as a link between genetic susceptibility and environmental factors co-fostering PD and PD-related pathogenesis (Gao *et al.*, 2011; McKenzie *et al.*, 2017).

Based on these findings and the lack of direct evidence proving that inflammation influences α -synO harmful activities or PD neuropathology, we investigated whether an induced or exacerbated inflammatory state potentiates α -synO effects in the acute mouse model previously described and PD phenotype in A53T mutated mice, respectively. To this goal we have developed two different “double-hit” mouse models. The former is an acute “double-hit” model based on the peripheral administration of LPS and the subsequent ICV injection of α -synOs. The latter is a Tg “double-hit” mouse model, which is based on the peripheral administration of LPS in either NTG or A53T mice. Of note, in this second model the two challenges are represented by the pathologic genetic PD-related background and the inflammatory stimulus LPS. For this study we have performed several experiments aimed at identifying the best experimental conditions. Firstly, we have identified a dose of α -synOs which did not affect memory in the NORT. Thus, C57BL/6 naïve mice were ICV injected with 0.5 μ M α -synOs (7.5 μ L) 2 hours before the familiarisation/sample phase and their memory performances assessed 24 hour later (Figure 42A). To identify a dose of LPS inducing the activation of both microglial and astroglial cells in the hippocampus without affecting memory performances, 1 or 2.5 mg/Kg were IP injected in C57BL/6 naïve mice.

One month later, their neuroinflammatory state was investigated through immunohistology, and memory performance were tested in the NORT (Figure 42B).

To address whether the peripherally LPS-induced neuroinflammation influences the effect of the ineffective dose of α -synOs, we have developed an acute “double-hit” mouse model. C57BL/6 naïve mice were IP treated with either Veh or 2.5 mg/Kg LPS. One month later, mice were ICV injected with either Veh or 0.5 μ M α -synOs and their memory performances tested 24 hours later in the NORT or in the Y-maze. In the NORT the ICV injection of Veh or 0.5 μ M α -synOs was performed 2 hours before the familiarisation/sample phase, and memory was investigated 24 hours later the ICV treatment. Immunohistological assessment of the neuroinflammatory response in the hippocampus was carried out 24 hours after the ICV injection (Figure 42C).

To elucidate whether the peripherally LPS-induced neuroinflammation influences cognitive and motor performances in a Tg PD mouse model, A53T mice were treated with a single IP injection of Veh or 2.5 mg/Kg LPS. Twenty-five days later, A53T and NTG mice were tested in cognitive tools (NORT, Y-maze and MWM) and in motor tools (open field and Beam walk test). At the end of behavioural experiments, a group of mice was sacrificed and both hippocampal neuroinflammation and dopaminergic neurodegeneration in the SNpc were evaluated through immunohistology (Figure 42D).

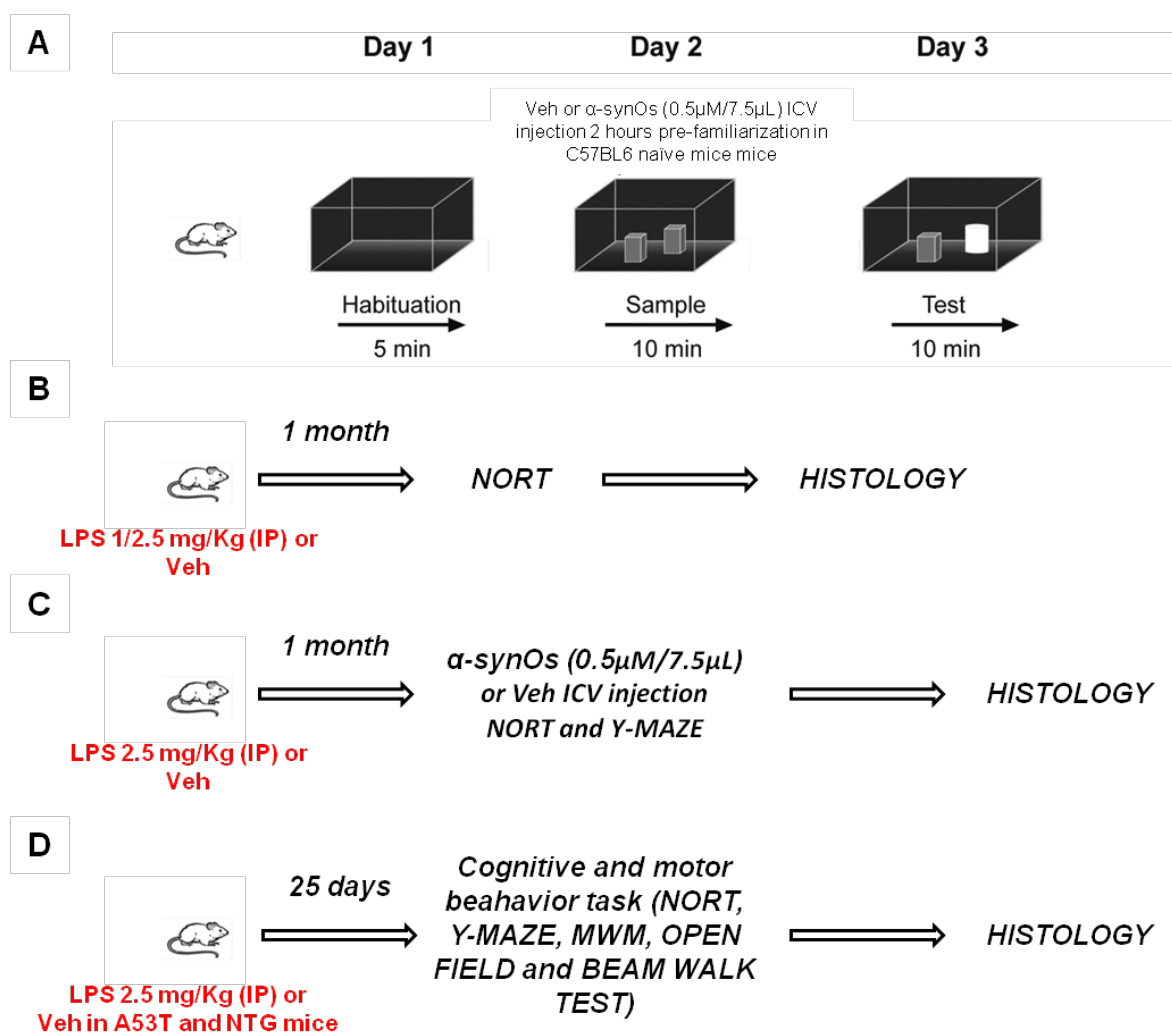


Figure 42. Schematic representation of the *in vivo* experiments. (A) Veh or 0.5 μ M α -synOs ICV injected in C57BL/6 naïve mice 2 hours pre-sample phase of the NORT. (B) Veh, 1 or 2.5 mg/Kg LPS IP injection in C57BL/6 naïve mice one month before cognitive and histological characterisation. (C) 2.5 mg/Kg LPS or Veh IP injected in C57BL/6 naïve mice one month before the ICV injection of Veh or 0.5 μ M α -synOs. 24 hours after the ICV treatment mice were tested in the test phase of NORT or in the Y-maze. (D) 2.5 mg/Kg LPS or Veh IP injected in either NTG or A53T mice. Starting 25 days after treatment mice were behaviourally characterised for their cognitive and motor performances in several behavioural tasks.

9.2 Results

9.2.1 LPS-preconditioning enhances α -synO-induced toxicity in primary hippocampal neuronal cultures

To assess whether inflammation would influence α -synO cytotoxicity, we initially exposed primary hippocampal neurons to α -synOs (1, 5 and 10 μ M) to identify a dose of oligomers ineffective in inducing neuronal death. We found that, upon 48 hours exposure, α -synOs induced cellular damage at the two highest doses (Figure 43), leading to a significant increase in the amount of LDH released in the cell medium (% of Veh mean \pm SEM; 132.2 \pm 6.4 and 137.3 \pm 1.6, respectively; * p <0.05 and ** p <0.01, One-way ANOVA followed by Dunnet's test). In contrast, since the LDH released by neurons upon exposure to 1 μ M α -synOs was comparable to Veh-treated cells (100.0 \pm 9.40 and 113.7 \pm 10.3, respectively), we demonstrate that 1 μ M α -synOs did not affect cell survival.

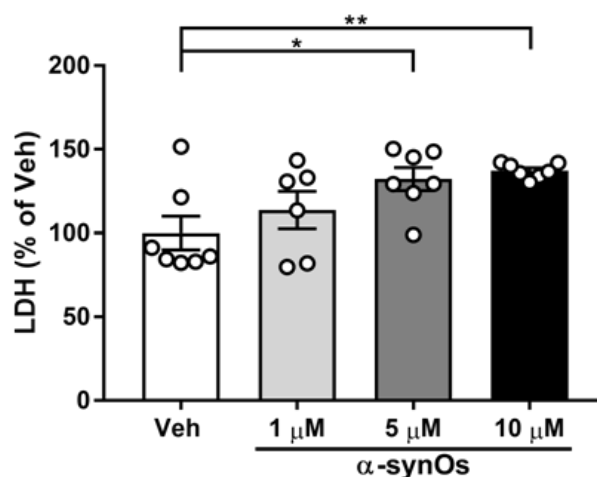


Figure 43. α -synO-mediated neuronal damage in primary hippocampal cell cultures. (A) Scatter plots and bars (mean \pm SEM) of the LDH percentage of primary hippocampal cultures exposed for 48 hours to Veh (n=7 wells), or 1 μ M (n=6 wells), 5 μ M (n=7 wells) and 10 μ M (n=7 wells) α -synOs (One-way ANOVA found a significant effect of *treatment*; $F_{2,23}=4.70$, $P=0.011$; * p <0.05 and ** p <0.01, Dunnet's test).

Once characterised 1 μ M as ineffective dose, we evaluated whether the neuronal culture pre-conditioning with the inflammatory stimulus LPS could influence neuronal susceptibility to α -synOs. Thus, primary hippocampal cultures were exposed to an inactive dose of LPS (0.5

$\mu\text{g/mL}$) for 24 hours. After LPS wash-out, we treated neurons for 24 and 48 hours with α -synOs (1 μM), then we assessed the cell toxicity through LDH assay. At both time points, we found that the 24-hour LPS pre-conditioning enhanced primary hippocampal cell susceptibility to α -synOs. In fact, as shown in figure 44A and B, while LPS 0.5 $\mu\text{g/mL}$ or 1 μM α -synOs did not affect neuronal survival *per se* at both time points tested (LDH 24 hours: 97.51 ± 1.50 n=8 wells and 98.52 ± 1.13 n=8 wells, respectively; 48 hours: 109.30 ± 6.50 n= 8 wells and 139.59 ± 11.82 n= 9 wells, respectively), which was comparable to Veh-treated cells (LDH 24 hours: 100.00 ± 2.31 , n=9 wells; 48 hours: 100.00 ± 3.29 , n=9 wells), cell death was significantly higher in neurons treated with both 0.5 $\mu\text{g/mL}$ LPS and 1 μM α -synOs (LDH 24 hours: 125.56 ± 7.13 , n=7 wells; 48 hours: 185.13 ± 15.16 , n=7 wells; ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, Two-way ANOVA followed by Tukey's test). Therefore, we herein demonstrate that LPS pre-conditioning of hippocampal neurons potentiates α -synOs detrimental effects *in vitro*.

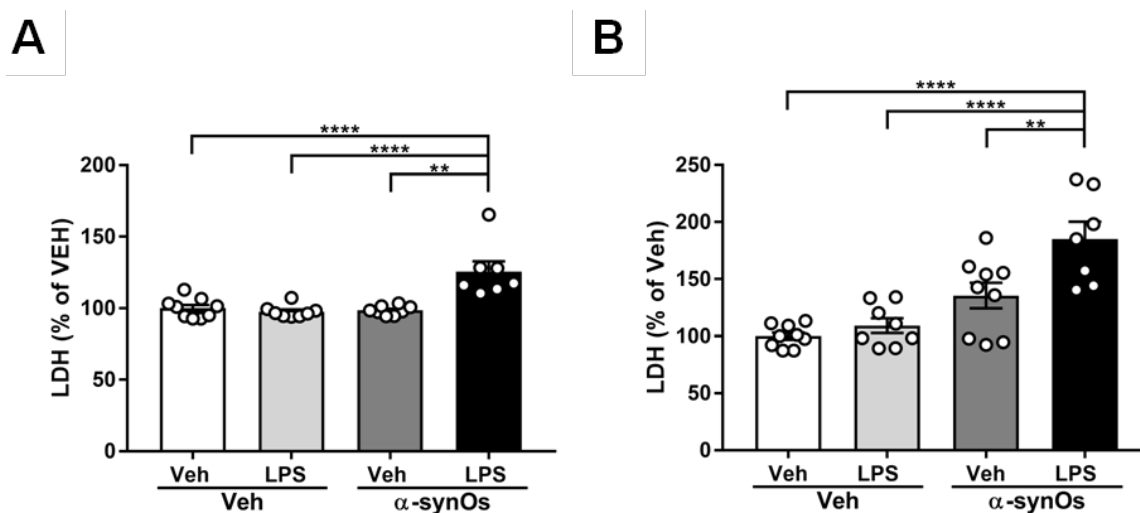


Figure 44. LPS pre-conditioning enhances α -synO-mediated neuronal damage in primary hippocampal cell cultures. (A) Scatter plots with bars (mean \pm SEM) of the LDH percentage of primary hippocampal cultures pre-conditioned with 0.5 $\mu\text{g/mL}$ LPS and then exposed to 1 μM α -synOs for 24 hours (Two-way ANOVA demonstrated a significant interaction $LPS \times \alpha$ -synOs: $F_{1,28}=17.67$, $P=0.0002$; *** $p < 0.001$ and **** $p < 0.0001$, Tukey's test). (B) Scatter plots with bars (mean \pm SEM) of the LDH percentage of primary hippocampal cultures pre-conditioned with 0.5 $\mu\text{g/mL}$ LPS (n=8 wells), and then exposed to 1 μM α -synOs for 48 hours (Two-way ANOVA showed

a significant interaction *LPS* \times *α -synOs*: $F_{1,28}=6.374$, $P=0.017$; ** $p<0.01$ and **** $p<0.0001$, Tukey's test).

9.2.2 Establishment of the two challenges for the “double-hit” experimental model

Based on our *in vitro* data, by exploiting the acute α -synO-induced mouse model described above, we ascertained whether a pre-established neuroinflammatory condition could potentiate α -synO detrimental effects *in vivo*. To such a purpose, we developed a “double-hit” acute mouse model exploiting two challenges. As initial approach, we identified the inactive α -synO concentration in C57BL/6 naïve mice in terms of memory impairment. As reported in chapters VI and VII and in our recent publications (La Vitola *et al.*, 2018; La Vitola *et al.*, 2019), 1 μ M ICV injection of α -synOs is able to induce memory impairment. Thus, we investigated the efficacy of the lower 0.5 μ M concentration in mice subsequently tested in the NORT. Mice receiving either Veh ($n=7$) or 0.5 μ M α -synOs ($n=8$) discriminated well between the familial and the novel object exhibiting comparable DIs (0.28 ± 0.02 and 0.31 ± 0.03 , respectively; Student's t -test $t_{13}=0.70$, $P=0.49$; Figure 45A).

Thereafter, we set up the LPS-induced neuroinflammatory treatment with the purpose of defining an LPS dose leading to a persistent neuroinflammatory response without inducing mouse suffering and cognitive deficiency. We injected IP C57BL/6 naïve mice with either 1 or 2.5 mg/Kg LPS ($n=7$ and $n=8$, respectively). Both LPS doses altered body growth (Figure 45B). Indeed, we observed a significant reduction in body weight three days after treatment compared to mice receiving Veh ($n=7$) (23.06 ± 0.54 g, 23.24 ± 0.76 g and 26.30 ± 0.30 g, respectively; **** $p<0.0001$, Two-way ANOVA followed by Tukey's test). Such an effect was transient, as the body weight recovered to Veh-treated mice one-month post-injection (28.34 ± 0.54 g, 29.45 ± 0.39 g and 29.93 ± 0.39 g, respectively). Thus, we selected the one-month post-injection as the time point for both LPS doses in order to establish the best treatment condition propaedeutic to evaluate the α -synO effects.

To choose the final LPS dose, we compared the memory performance and the extent of neuroinflammation at 1 and 2.5 mg/Kg. As shown in figure 45C, both LPS doses (n=4/group) induced hippocampal astrogliosis (marked by GFAP) compared to Veh-treated mice (n=4/group). The subsequent quantitative analysis (Figure 45D) of the percentage of GFAP-marked area (mean±SEM of Veh-treated mice) confirmed our qualitative result (1 mg/kg = 1119.39±1.99; 2.5 mg/kg = 144.59±2.4; Veh = 100±1.17; ***p<0.001 and****p<0.0001, One-way ANOVA followed by Tukey's test). Notably, we found that the GFAP expression was significantly increased in 2.5 mg/Kg LPS-treated mice compared to the lower dose (****p<0.0001, One-way ANOVA followed by Tukey's test).

In contrast to the astroglial activation, microglial cells were significantly activated in the hippocampus solely at the higher LPS dose (Figure 45C). Moreover, as shown in figure 45E, the quantitative analyses of the IBA1-marked area, confirmed a significant increase in the percentage of IBA1 expression (mean±SEM of Veh-treated mice) in mice receiving LPS 2.5 compared to both LPS 1 mg/Kg and Veh-treated animals (250.2±7.52, 133.19±23.99 and 100±7.01, respectively, n=4/group; **p<0.01 and ***p<0.001, One-way ANOVA followed by Tukey's test). Thus, our immunohistochemical analyses demonstrated that 2.5 mg/Kg LPS efficiently leads to a significant hippocampal glial response one month after treatment. We, thus, assessed the memory performance of LPS-treated mice in the NORT at the same time point (Figure 45F). As suggested by comparable DIs (mean±SEM), we demonstrated that neither 1 nor 2.5 mg/Kg LPS (0.26±0.11 and 0.27±0.10 respectively, n=8/group) affected memory compared to Veh-treated mice (DI=0.27±0.08, n=8).

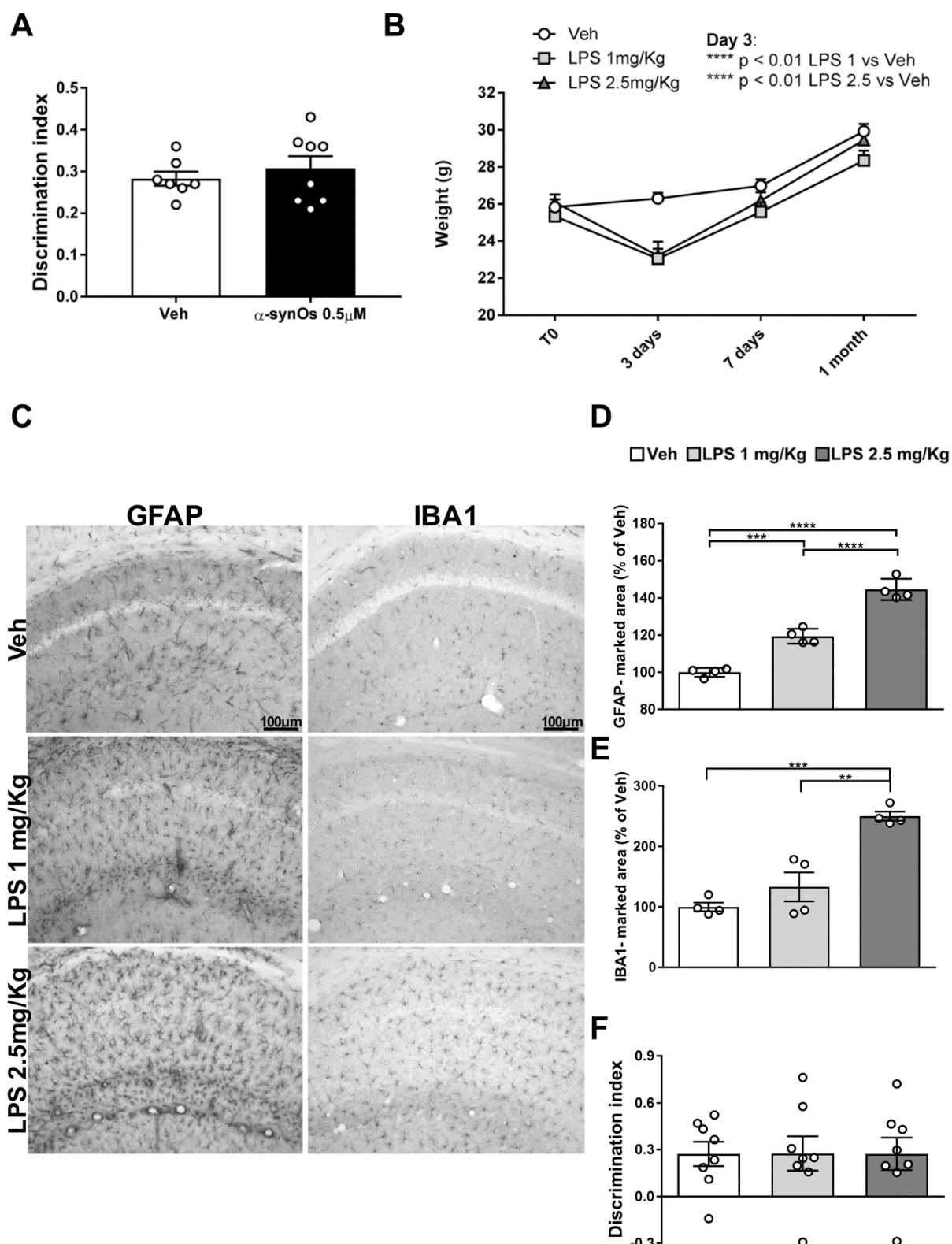


Figure 45. Identification of an α -synO dose ineffective in mediating memory damage in the NORT, and characterisation of the peripheral LPS-induced neuroinflammatory mouse model (A) Scatter plots and bars are the DI (mean \pm SEM) of C57BL/6 naïve mice ICV injected with Veh or 0.5 μ M α -synOs. (B) Body weight growth (mean \pm SEM) of mice receiving a single IP administration of Veh, 1 or 2.5 mg/Kg LPS at different time points (Two-way ANOVA found a significant interaction *time* \times *treatment* ($F_{6,57}=4.89$, $P=0.0004$; Day 3: **** $p<0.0001$, Tukey's test). (C)

Representative images of hippocampal astrocytosis (GFAP) and microgliosis (IBA1) one month after the IP administration of Veh, 1 or 2.5 mg/Kg LPS. **(D)** Scatter plots with bars (mean \pm SEM) are the quantitative analysis in the hippocampus of the percentage GFAP-marked area in Veh, 1 and 2.5 mg/Kg LPS treated mice (One-way ANOVA found a significant effect of *treatment*: $F_{2,9}=112.1$, $P<0.0001$; *** $p<0.001$ and **** $p<0.0001$, Tukey's test). **(E)** Scatter plots and bars (mean \pm SEM) are the quantitative analysis of the percentage IBA1-marked area in mice receiving Veh, 1 and 2.5 mg/Kg LPS (One-way ANOVA found a significant effect of *treatment*: $F_{2,9}=27.35$, $P=0.0001$; ** $p<0.01$ and *** $p<0.001$, Tukey's test), **(F)** Scatter plots and bars are the DI (mean \pm SEM) of mice receiving Veh, 1 or 2.5 mg/Kg LPS one month after treatment. One-way ANOVA did not find any significant effect of *treatment* ($F_{2,21}=0.0003$; $P=0.99$).

Collectively, our findings depict 0.5 μ M α -synOs and LPS 2.5 mg/Kg as valuable experimental conditions for our further investigation. Based on this, we have developed the “double-hit” acute model in order to test whether the presence of a peripherally pre-established neuroinflammatory state could influence α -synO effects at both cognitive and neuroinflammatory level.

9.2.3 Peripherally LPS-induced neuroinflammation enhances α -synO-mediated memory damage

One month after the LPS injection, C57BL/6 naïve mice were ICV injected with α -synOs (0.5 μ M/7.5 μ L) and their memory performance was assessed in the NORT (Figure 46). As demonstrated above, we confirmed that LPS 2.5 mg/Kg did not impair memory. In fact, LPS mice had a DI (mean \pm SEM) comparable to those receiving Veh (0.29 \pm 0.3 and 0.30 \pm 0.02, respectively; $n=7$ /group). Moreover, since mice treated with Veh+ α -synOs ($n=7$) had a DI comparable to that of Veh-treated animals (0.32 \pm 0.06 and 0.30 \pm 0.02, respectively), we confirmed again that no effects on memory performance were detectable upon 0.5 μ M α -synOs application. In contrast, we found that mice receiving both LPS and α -synOs ($n=7$) showed a significant impairment in their recognition memory having a DI significantly lower than Veh-injected mice (DI=-0.03 \pm 0.06; *** <0.001 , Two-way ANOVA followed by Tukey's test). Furthermore, we found that double-hit-treated mice displayed a DI

significantly lower than both Veh+ α -synOs and LPS+Veh (0.32 ± 0.06 and 0.29 ± 0.3 , respectively; *** $p<0.001$, Two-way ANOVA followed by Tukey's test).

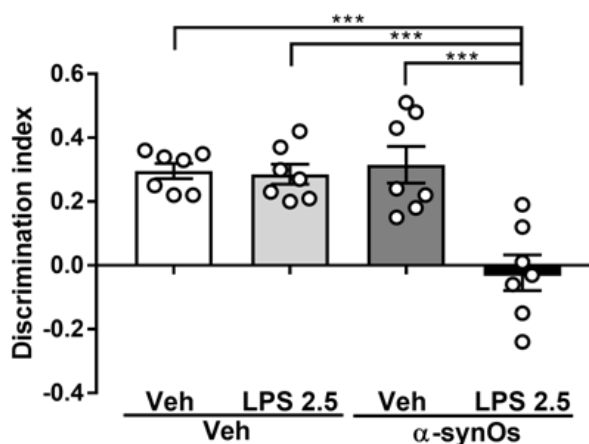


Figure 46. 0.5 μ M α -synO detrimental effects on recognition memory is enhanced by the peripheral 2.5 mg/Kg LPS pre-treatment. Scatter plots with bars (mean \pm SEM) of the DI of mice ICV injected with either Veh or 0.5 μ M α -synOs one month after a single IP administration of either Veh or 2.5 mg/Kg LPS (Two-way ANOVA found a significant interaction *LPS 2.5* \times *α -synOs*: $F_{1,24}=13.51$; $P=0.0012$; ** $p<0.01$ and **** $p<0.001$, Tukey's test).

To further assess the role of the pre-established LPS-induced neuroinflammation in promoting α -synO detrimental effects on memory, we also evaluated the short-term spatial working memory through the Y-maze test. One month after the 2.5 mg/Kg LPS treatment, mice were ICV injected with α -synOs (0.5 μ M/7.5 μ L) and their spatial working memory was tested 24 hours later. As shown in figure 47A, mice receiving LPS+ α -synOs ($n=6$) had a significant impairment in their spatial working memory having a lower spontaneous alternation behaviour (% mean \pm SEM) compared to Veh+Veh- ($n=6$), LPS+Veh- ($n=8$) and Veh+ α -synOs-treated mice ($n=6$), (52.65 ± 5.45 , 86.45 ± 3.79 , 85.66 ± 3.60 and 82.85 ± 3.88 , respectively; *** $p<0.0001$ and *** $p<0.001$, Two-way ANOVA followed by Tukey's test). Of note, the impaired spontaneous alternation behaviour was not due to an impairment of mouse motor performances. In fact, as shown in figure 47B, all treated mice had a comparable number of total arm entries.

Thus, our findings demonstrated that the peripherally LPS-induced neuroinflammation is able to promote the harmful activities of *per se* ineffective α -synOs on two different memory domains such as the long-term recognition memory and the short-term spatial working memory.

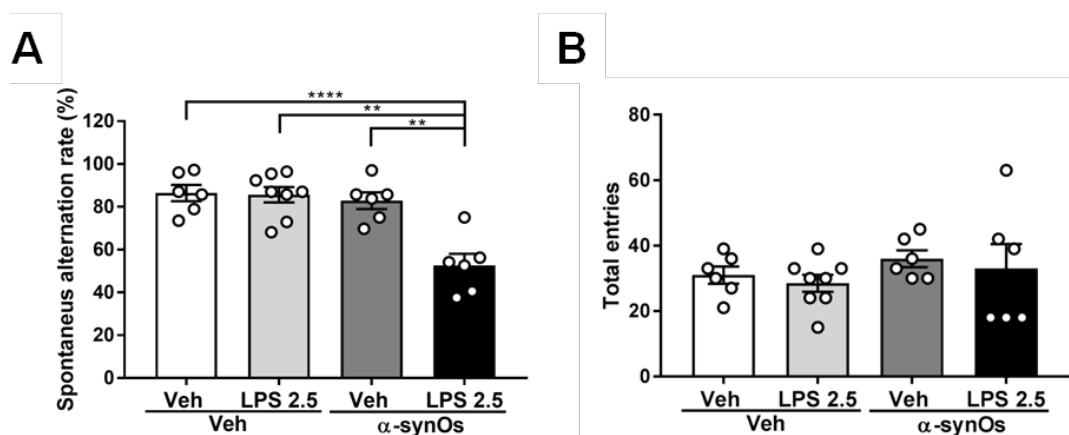


Figure 47. Peripheral 2.5 mg/Kg LPS pre-treatment enhances 0.5 μ M α -synO detrimental effects on spatial working memory. (A) Scatter plots and bars (mean \pm SEM) are the percentage of the spontaneous alternation behaviour in the Y-maze of mice ICV injected with either Veh or 0.5 μ M α -synOs one month after a single IP administration of either Veh or 2.5 mg/Kg LPS (Two-way ANOVA found a significant interaction $LPS \times \alpha$ -synOs: $F_{1,22}=12.18$; $P=0.0024$; *** $p<0.001$ and **** $p<0.0001$, Tukey's test). (B) Scatter plots and bars (mean \pm SEM) are the total arms entries of mice tested in the Y-maze. Two-way ANOVA did not find a significant interaction $LPS \times \alpha$ -synOs ($F_{1,22}=0.036$; $P=0.95$) and a significant effect of both LPS -treatment ($F_{1,22}=0.43$; $P=0.52$) and of α -synO-treatment ($F_{1,22}=1.282$; $P=0.27$).

9.2.4 Microglial cells and astrocytes differentially respond in the “double-hit” acute mouse model

Since we have previously demonstrated that a single ICV injection of α -synOs (1 μ M/7.5 μ L) leads to hippocampal gliosis in tight association with memory damage (La Vitola *et al.*, 2018; chapter VII), we have investigated whether the peripherally pre-established LPS-induced glial cell activation would influence the action of α -synOs on both microglial and astroglial cells in our new “double-hit” acute model. At the end of the NORT, we sacrificed

mice and performed immunohistochemical analyses. As shown in figure 48A, mice pre-treated with LPS and ICV injected with α -synOs showed IBA1⁺ cells characterised by an enlargement of their soma and with an amoeboid shape, typical features of activated microglia. Of note, we confirmed our qualitative analysis by the quantification of the percentage of IBA1-marked area (Figure 48B). Specifically, mice receiving LPS+ α -synOs (n=7) had an IBA1-marked area (% of Veh mean \pm SEM) significantly higher than animals treated with Veh+Veh (n=7), LPS 2.5 mg/Kg+Veh (n=7) and Veh+ α -synOs (n=6) (338 ± 34.39 , 100 ± 18.25 , 227.25 ± 23.36 and 103.14 ± 19.71 , respectively; * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$, Two-way ANOVA followed by Tukey's test).

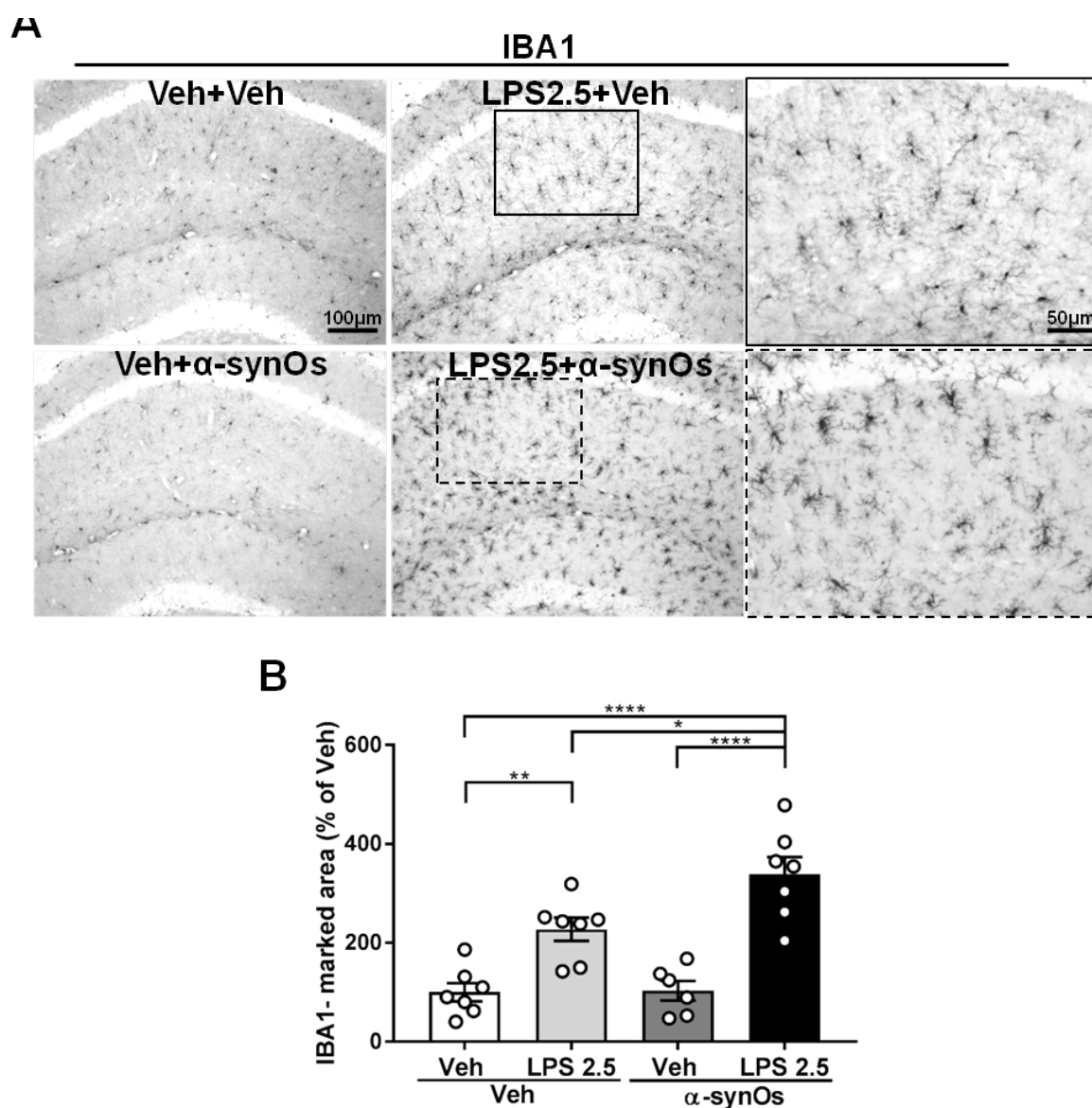


Figure 48. Peripherally LPS pre-activated microglial cells are primed to α -synOs and potentiate their activation state. (A) Representative images showing the activation of microglial cells (IBA1) in the CA1 region of mice ICV injected with either Veh or 0.5 μ M α -synOs one month after a single IP administration of either Veh or 2.5 mg/Kg LPS. (B) Scatter plots and bars (mean \pm SEM) are the quantitative analysis in the hippocampus of the percentage IBA1-marked area of the different experimental group considered (Two-way ANOVA revealed a significant interaction $LPS \times \alpha$ -synOs: $F_{1,23}=4.615$, $P=0.04$; * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$, Tukey's test).

To further characterise the microglial cell phenotype in our “double-hit” model, we measured the expression of CD16/32, representative of an M1 pro-inflammatory microglial phenotype (Subramaniam and Federoff, 2017). We found that CD16/32 was poorly expressed in Veh+Veh, Veh+ α -synOs and LPS+Veh-treated mice. In contrast, it was dramatically increased in mice receiving LPS+ α -synOs (Figure 49A). Accordingly, the quantitative analysis of the CD16/32-marked area (% mean \pm SEM) normalised on the hippocampal area, confirmed our qualitative results (Figure 49B). Specifically, we found that LPS+ α -synOs ($n=5$) had a significant higher CD16/32-marked area than Veh+Veh, LPS+Veh and Veh+ α -synOs ($n=4$ /group) (3.19 ± 0.79 , 0.11 ± 0.04 , 0.28 ± 0.14 and 0.36 ± 0.14 , respectively; ** $p<0.01$, Two-way ANOVA followed by Tukey's test).

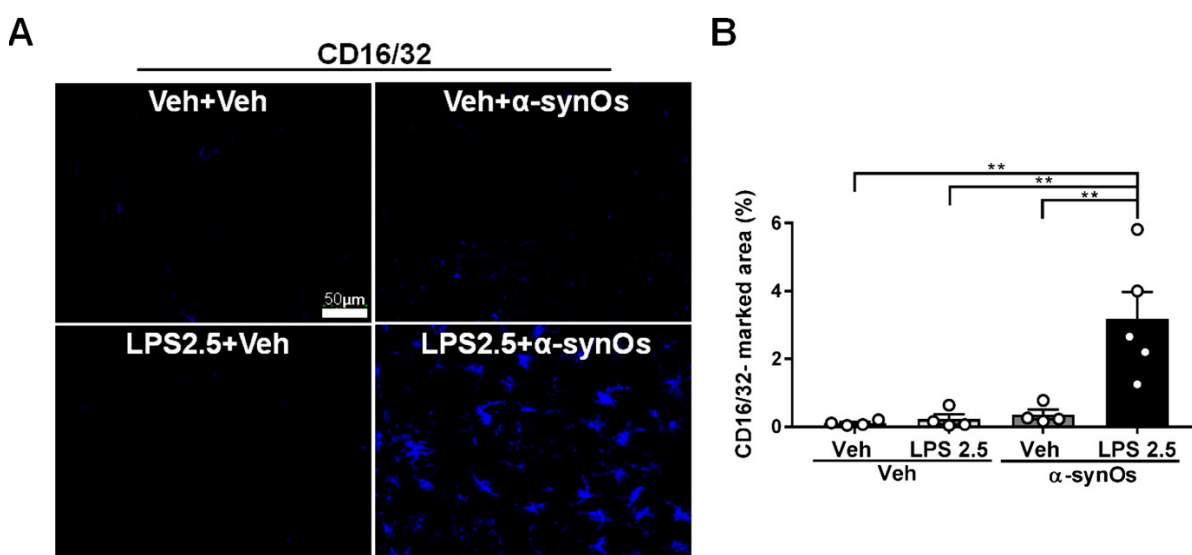


Figure 49. Peripherally LPS pre-activated microglial cells are primed to α -synOs and potentiate their activation state acquiring an M1 pro-inflammatory phenotype. (A) Representative immunofluorescence images of the M1 pro-inflammatory phenotype marker

CD16/32 in the CA1 hippocampal region of mice ICV injected with either Veh or 0.5 μ M α -synOs one month after a single IP administration of either Veh or 2.5 mg/Kg LPS. **(B)** Scatter plots and bars (mean \pm SEM) are the quantitative analysis of the hippocampal normalised percentage CD16/32-marked area of the different experimental group considered (Two-way ANOVA revealed a significant interaction *LPS* \times *α -synOs*: $F_{1,23}=4.615$, $P=0.04$; $**p<0.01$, Tukey's test).

To confirm that CD16/32⁺ were indeed microglial cells, we co-localised CD16/32 with the microglial marker IBA1. As shown in figure 50, in mice receiving LPS+ α -synOs, CD16/32 completely co-localised with IBA1. Thus, confirming that CD16/32⁺ were microglial cells.

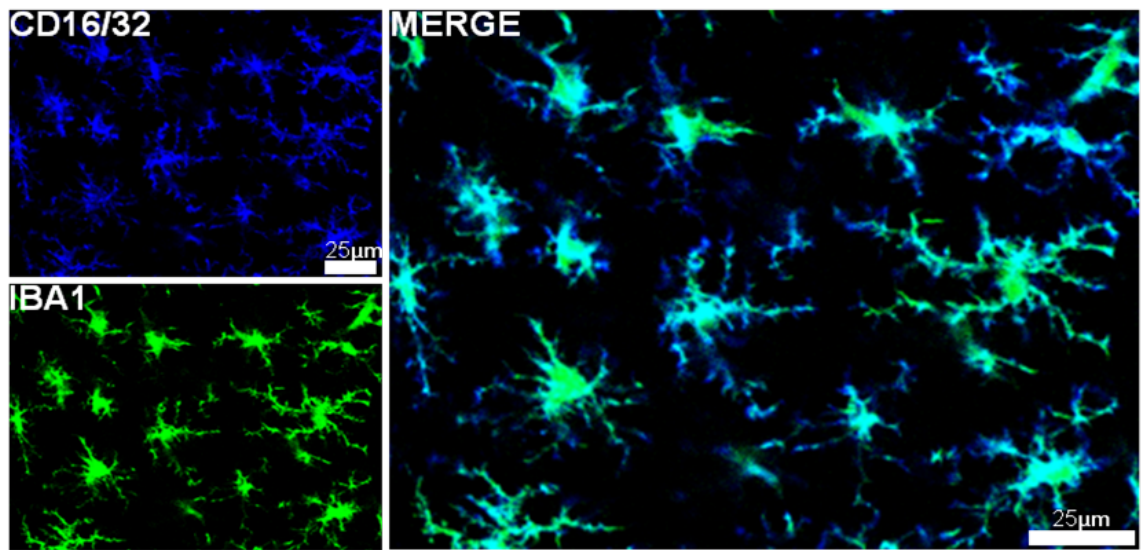


Figure 50. CD16/32⁺ cells in “double-hit” mice are microglial cells. Representative immunofluorescence images showing the complete colocalization of the M1 marker CD16/32 and the pan-microglial marker IBA1 in the hippocampal CA1 region of LPS2.5+ α -synO treated mice

In contrast to the synergistic effect of LPS and α -synOs on microglial cell activation, astrocytes differently responded to the “double-hit” stimulation. In fact, we found a significant reduction in the expression of the astroglial marker GFAP. In particular, while α -synOs alone did not alter the expression of GFAP, and LPS induced a significant increase when compared to mice receiving Veh, “double-hit” mice surprisingly showed a reduction in the GFAP-immunoreactivity (Figure 51A and B). The GFAP quantification (% of Veh mean \pm SEM; Figure 51C) found that the percentage of GFAP-marked area was increased in LPS-treated mice compared to mice receiving Veh (134.3 \pm 5.81 and 100 \pm 4.35 respectively;

n=7/group; *p<0.05, Two-way ANOVA followed by Tukey's test), but was significantly reduced in LPS+ α -synO-treated mice (65.69 ± 4.51 , n=7) compared to all the other experimental groups considered (Veh+Veh: 100 ± 4.35 ; Veh+ α -synOs: 96.16 ± 13.46 , n=6 and LPS 2.5mg/Kg+Veh: 134.3 ± 5.81 ; *p<0.05 and ****p<0.0001, Two-way ANOVA followed by Tukey's test).

In order to establish whether the GFAP reduction was attributable to cell loss, we counted the number of GFAP⁺ cells (% of Veh mean \pm SEM; Figure 51D), which was unchanged. Thus, this result suggested that the dampening of the GFAP-immunoreactivity might be the result of a reduced GFAP expression and astrocyte branches rather than cell loss.

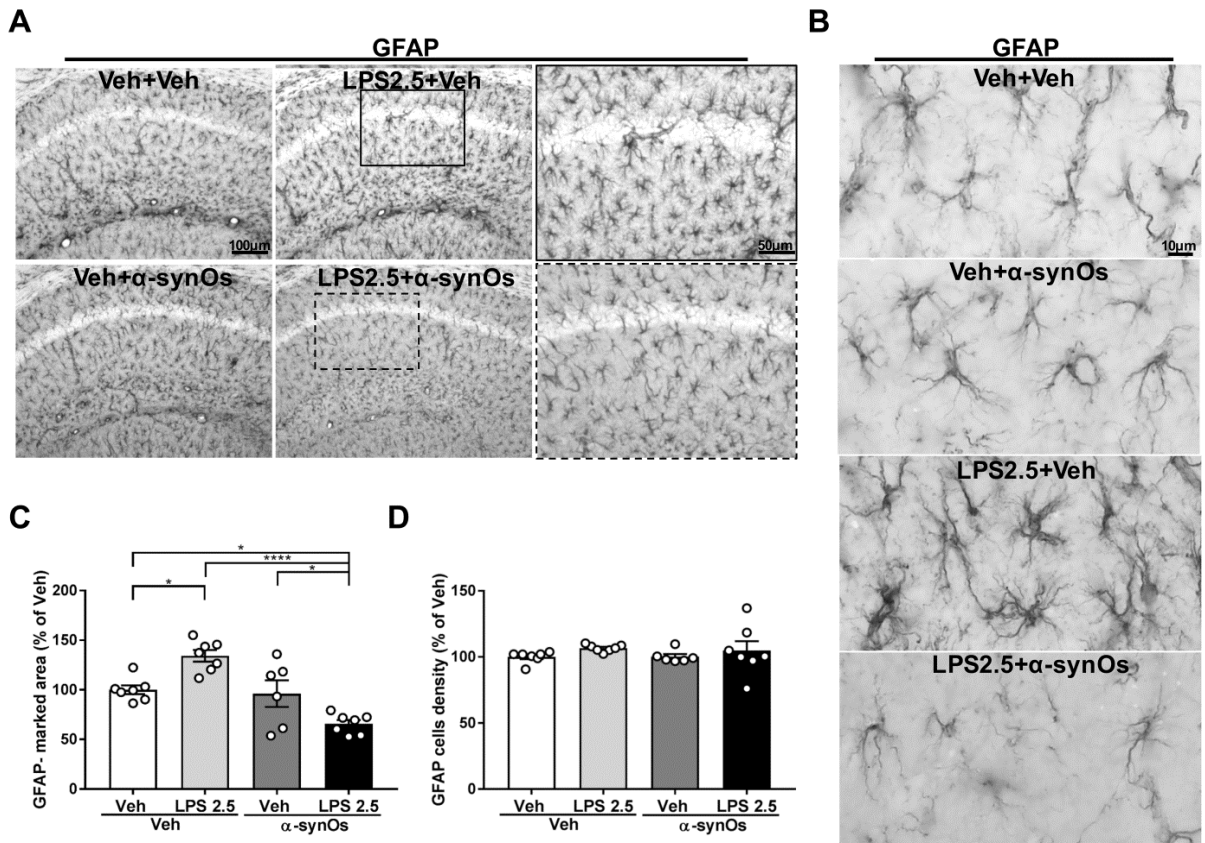


Figure 51. Alpha-synO challenge triggers the atrophy of peripherally LPS pre-activated astrocytes. (A and B) Representative images showing astroglial cells (GFAP) in the CA1 region of mice ICV injected with either Veh or 0.5 μ M α -synOs one month after a single IP administration of either Veh or 2.5 mg/Kg LPS. **(C)** Scatter plots with bars (mean \pm SEM) are the quantitative analysis of the percentage GFAP-marked area in the hippocampus of the different experimental groups considered (Two-way ANOVA found a significant interaction *LPS* \times *α -synOs*: $F_{1,23}=19.64$, $P=0.0002$; *p<0.05, ****p<0.0001, Tukey's test). **(D)** Scatter plots and bars (mean \pm SEM) are the quantitative analysis of the percentage hippocampal GFAP⁺ cell density of the different experimental

groups investigated. Two-way ANOVA did not find any significant interaction *LPS* \times *α -synOs* ($F_{1,23}=0.065$; $P=0.80$).

9.2.5 Peripheral administration of LPS negatively influences cognitive performances in the A53T PD-related transgenic mouse model

Since through our acute “double-hit” mouse model we demonstrated that the peripherally LPS-induced neuroinflammation increases the susceptibility to α -synO detrimental actions on both memory and glial activation, we further investigated whether the harmful effects of the induced neuroinflammation are detectable also in the more complex scenario provided by a Tg mouse model of PD. To this purpose, eight months old A53T mice and their age-matched NTG were IP injected with either Veh or 2.5 mg/Kg LPS and, starting from day 25 after treatment, we behaviourally characterised them to assess both memory and motor performances. Thereafter, mice were sacrificed and their hippocampal gliosis, as well as dopaminergic neurodegeneration in the SNpc, were addressed.

We found that LPS aggravated memory performance of A53T mice tested in the NORT. In fact, as shown in figure 52A, while NTG mice treated with either Veh or LPS discriminated well between the novel and the familial object, showing a comparable DI (mean \pm SEM) (0.26 ± 0.02 and 0.30 ± 0.01 , respectively; $n=7$ /group), A53T+Veh mice did not (0.11 ± 0.01 , $n=7$; $**<0.01$, Two-way ANOVA followed by Tukey’s test). Intriguingly, we found that the memory deficiency of A53T mice was further exacerbated by the LPS administration (DI= -0.004 ± 0.05 , $n=7$; $*p<0.05$ and $****p<0.0001$, Two-way ANOVA followed by Tukey’s test). To further prove the exacerbating effect of the LPS-induced neuroinflammation at cognitive level in A53T mice, we tested mice also in spatial memory tasks.

Spatial memory was tested in both the Y-maze measuring the spontaneous alternation behaviour (% mean \pm SEM), and in the MWM for learning and memory assessment.

Despite the recognition memory impairment, spontaneous alternation behaviour (Figure 52B) was not damaged in Veh-treated A53T mice (66.37 ± 4.48), which was comparable to

that of NTG (+ Veh: 74.44 ± 2.98 ; + LPS: 74.44 ± 2.98). In contrast, a significant impairment was detectable in LPS-treated A53T mice (42.73 ± 5.10 ; $**p < 0.01$ and $***p < 0.001$, Two-way ANOVA followed by Tukey's test). To rule out possible motor deficits underlying the spontaneous alternation behaviour impairment, we analyzed the total mouse arm entries in the Y-maze. Two-way ANOVA neither found a significant interaction *genotype* \times *LPS* 2.5 ($F_{1,24} = 0.002$; $P = 0.96$) nor a significant effect of either *genotype* ($F_{1,24} = 1.46$; $P = 0.24$) or *LPS* 2.5 ($F_{1,24} = 1.684$; $P = 0.21$).

When mice were tested in the MWM (Figure 52C), NTG receiving Veh progressively learnt platform position and reduced their latency to reach it (mean \pm SEM) (d1: 34.4 ± 2.8 ; d5: 11.8 ± 1.4 ; $*p < 0.05$, Three-way ANOVA followed by Tukey's test) as well as NTG+LPS (d1: 41.8 ± 5.5 ; d5: 13.6 ± 2.1 ; $***p < 0.001$, Three-way ANOVA followed by Tukey's test) and A53T receiving Veh (52.4 ± 3.5 sec and 14.6 ± 1.5 ; $***p < 0.001$, Three-way ANOVA followed by Tukey's test). In contrast, LPS-treated A53T animals did not and they showed a similar latency throughout the learning phase (d1: 55.0 ± 3.1 ; d5: 44.8 ± 4.7). Furthermore, we specifically analyzed the differences between A53T mouse groups through a Student's t-test for days 3 to 5 and, we found a significant longer latency for LPS-treated A53T mice at all three training days compared to A53T mice receiving Veh (d3: $t_{12} = 2.53$, $*p = 0.03$; d4: $t_{12} = 3.2$, $**p = 0.008$; d5: $t_{12} = 6.1$, $****p < 0.0001$). Besides hampering A53T learning, LPS impaired also their ability to recall platform location in the probe test phase (Figure 52D and E). Specifically, A53T+LPS spent a lower time (% mean \pm SEM) in the target quadrant compared to A53T mice receiving Veh and NTG animals treated with either Veh or LPS (19.50 ± 5.15 , 47.74 ± 3.85 , 47.78 ± 4.39 and 43.98 ± 4.02 respectively; $**p < 0.01$ and $***p < 0.001$, Three-way ANOVA followed by Tukey's test). Consistently, in the opposite quadrant, A53T mice receiving LPS spent a longer time (33.00 ± 5.41) compared to Veh-treated A53T mice (12.50 ± 1.41) and NTG animals receiving either Veh or LPS (9.60 ± 2.76 and 13.20 ± 2.31 respectively; $**p < 0.01$ and $***p < 0.001$, Three-way ANOVA followed by

Tukey's test). Of note, lack of spatial learning and memory deficits in A53T mice at the age tested is in accordance with recent data showing that in heterozygous A53T mice, these effects become detectable at 12 months of age (Singh *et al.*, 2019).

Collectively our findings demonstrate that the peripheral administration of LPS in A53T mice dampens their memory performances in diverse domains.

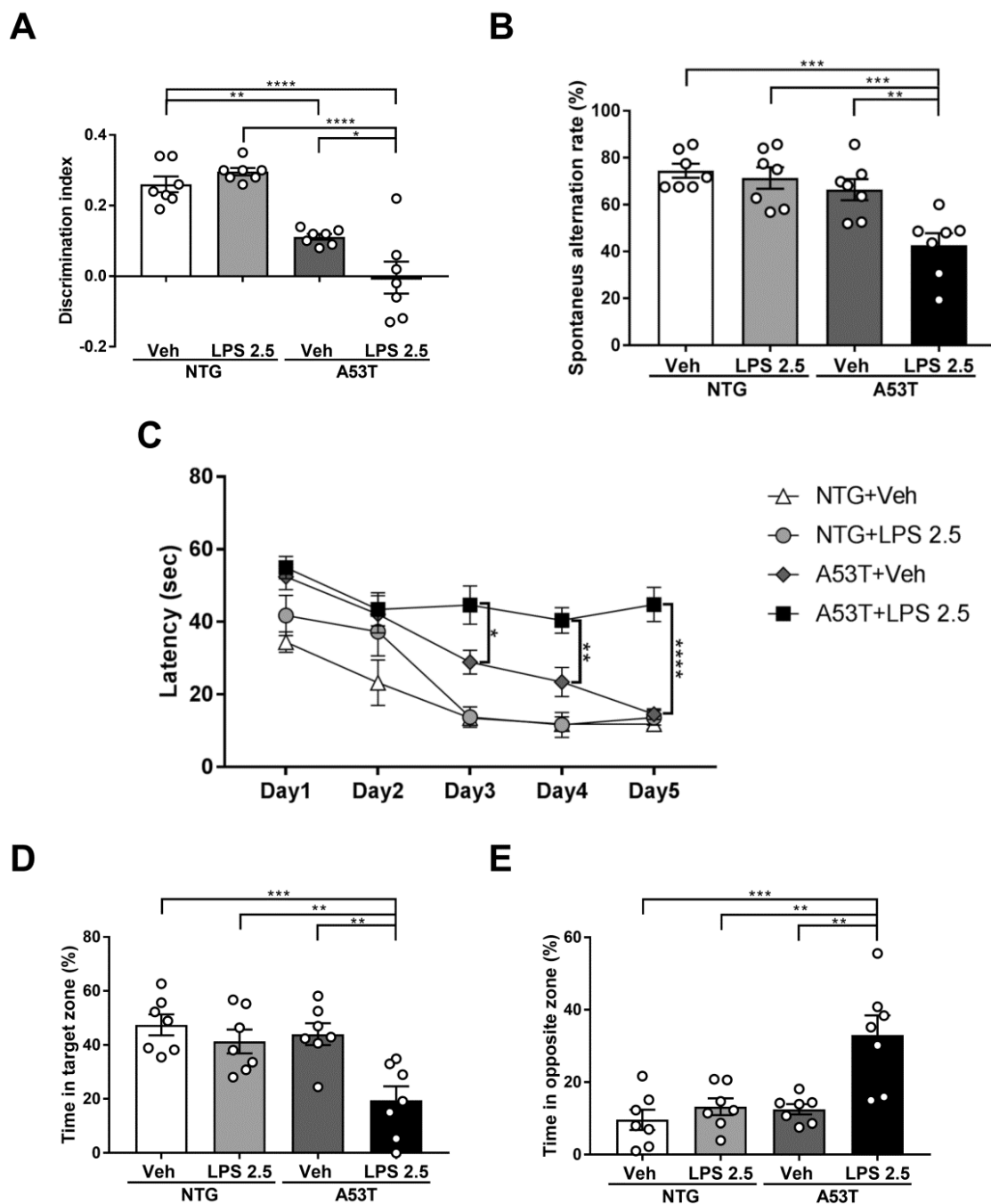


Figure 52. Peripheral LPS worsens cognitive functions in A53T mice. (A) Scatter plots and bars (mean±SEM) are the DI of both NTG and A53T mice receiving a single IP administration of either

Veh or 2.5 mg/Kg LPS (Two-way ANOVA that found a significant interaction *genotype x LPS*: $F_{1,24}=8.315$, $P=0.008$; * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$, Tukey's test). **(B)** Scatter plots and bars (mean \pm SEM) are the percentage of the spontaneous alternation behaviour of mice tested in the Y-maze (Two-way ANOVA found a significant interaction *genotype x LPS*: $F_{1,24}=5.587$, $P=0.027$; ** $p<0.01$ and *** $p<0.001$, Tukey's test). **(C)** Latency in finding the hidden platform (mean \pm SEM) during the five training days of the different experimental groups tested in the MWM (Three-way ANOVA found a significant interaction *time x genotype x LPS*: $F_{14,120}=29.19$, $P=0.0001$). Focusing on differences between A53T mouse groups through a Student's t-test for days 3 to 5 we found significant longer latencies for A53T mice treated with LPS at all 3 training days (d3: $t_{12} = 2.53$, * $p = 0.03$; d4: $t_{12} = 3.2$, ** $p = 0.008$; d5: $t_{12} = 6.1$, **** $p<0.0001$). **(D and E)** Scatter plots and bars (mean \pm SEM) are the percentage of total time spent in the target and opposite zone during the probe phase by the different experimental groups investigated Target quadrant: two-way ANOVA demonstrated a significant interaction *genotype x LPS* ($F_{1,24}=4.375$, $P=0.047$; ** $p<0.01$ and *** $p<0.001$, Tukey's test). Opposite quadrant: two-way ANOVA demonstrated a significant interaction *genotype x LPS* ($F_{1,24}=6.459$, $P=0.018$; ** $p<0.01$ and *** $p<0.001$, Tukey's test).

9.2.6 Peripheral LPS induces a different response of microglial and astroglial cells in the A53T PD-related transgenic mouse model

Since in the “double-hit” acute model we have demonstrated a different effect on glial cell activation, we further addressed whether these outcomes occurred also in A53T mice.

Through IBA1 immunostaining we evaluated microgliosis in the hippocampus (Figure 53A). We found an increased IBA1-immunoreactivity in NTG mice receiving LPS as well as in either Veh- or LPS-treated A53T mice compared to Veh-treated NTG. Quantitatively, the analysis of the hippocampal normalised IBA1-marked area (% mean \pm SEM) confirmed our qualitative observations (Figure 53B). Specifically, LPS-treated NTG ($n=5$) and A53T mice receiving Veh ($n=5$) had a significantly higher IBA1-marked area than Veh-treated NTG ($n=6$) (2.66 ± 0.13 , 2.51 ± 0.12 and 1.96 ± 0.09 , respectively; * $p<0.05$ and ** $p<0.01$, Two-way ANOVA followed by Tukey's test). Notably, in accordance with the acute “double-hit” model, LPS treatment further elicited microglial activation. In fact, A53T mice receiving 2.5 mg/Kg LPS ($n=5$) had a higher IBA1 expression with respect to all the other experimental groups (3.74 ± 0.16 ; **** $p<0.0001$, Two-way ANOVA followed by Tukey's test).

As aforementioned, microglia and astroglia differentially responded to the LPS and α -synO challenges in the “double-hit” acute model. Thus, we investigated in A53T mice how astrocytes responded to the peripheral LPS stimulation (Figure 53C). While both NTG mice receiving LPS and A53T animals receiving Veh (n=5/group) had an increased GFAP expression compared to Veh-treated NTG mice (n=6), A53T+LPS mice (n=5) did not. Quantitative analysis (Figure 53D) confirmed that NTG mice treated with LPS and A53T mice receiving Veh had a significantly higher GFAP-marked area (% mean \pm SEM) than the NTG Veh-treated group (11.09 \pm 0.40, 11.51 \pm 0.26 and 9.49 \pm 0.36, respectively; *p<0.05 and **p<0.01, Two-way ANOVA followed by Tukey’s test). In contrast, astroglial cell activation in A53T mice receiving LPS was comparable to NTG mice treated with Veh. To be noticed that, in line with results we obtained in the “double-hit” acute model, although the GFAP-marked area in A53T mice receiving LPS was not significantly lower than NTG+Veh-treated mice, it was significantly lower than both LPS-treated NTG and Veh-treated A53T mice (9.24 \pm 0.50; *p<0.05 and **p<0.01, Two-way ANOVA followed by Tukey’s test).

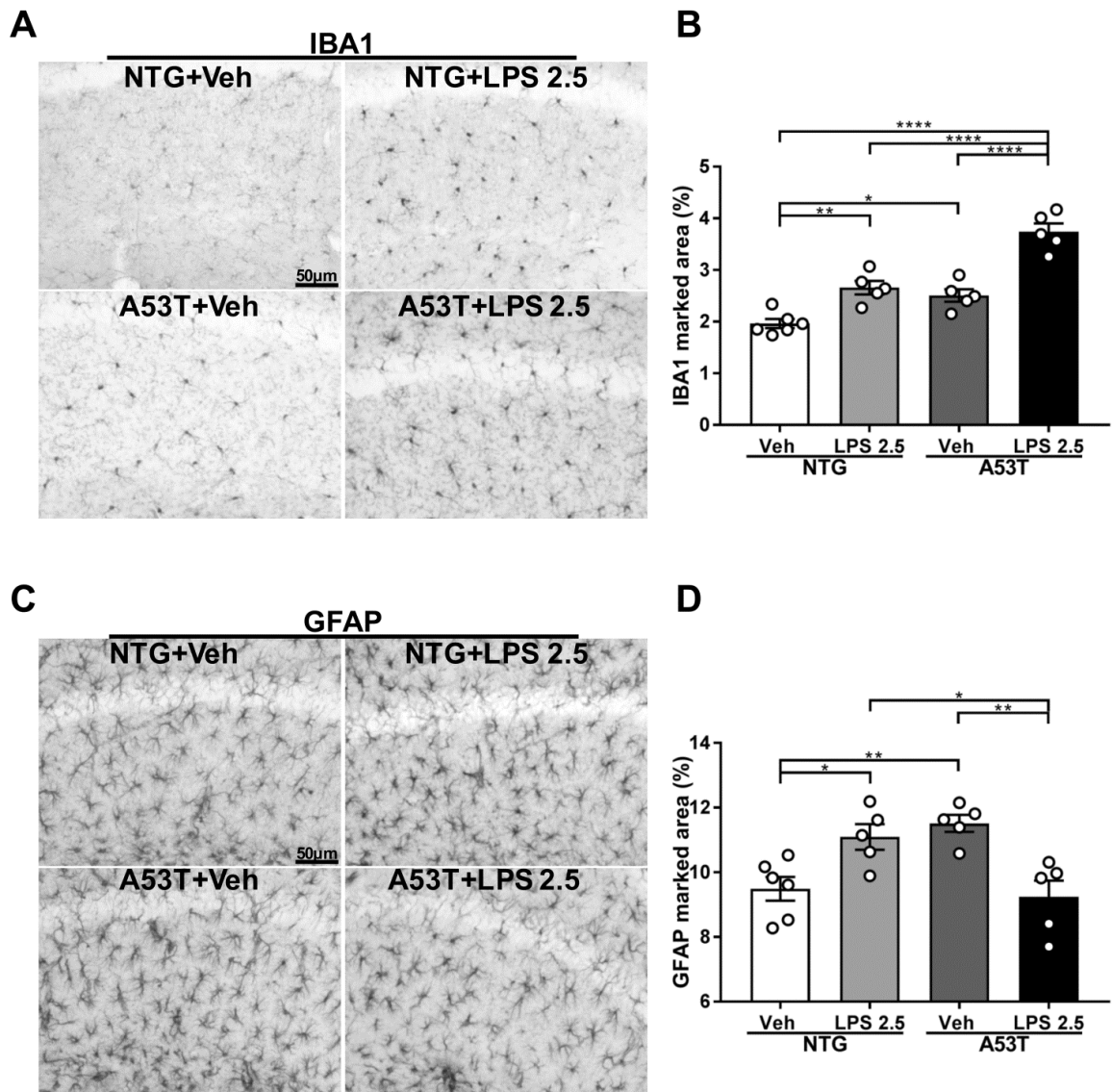


Figure 53. Microglial and astroglial cells differentially respond to the peripheral 2.5 mg/Kg LPS challenge in A53T mice. (A) Representative images showing the activation of microglial cells (IBA1) in the hippocampal CA1 region of both NTG and A53T mice treated with a single IP administration of either Veh or 2.5 mg/Kg LPS. (B) Scatter plots and bars (mean±SEM) are the quantitative analysis of the hippocampal normalised percentage IBA1-marked area of the different experimental group considered (Two-way ANOVA found a significant interaction *genotype x LPS* 2.5: $F_{1,17}=4.59$, $P=0.047$; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$, Tukey's test). (C) Representative images showing astroglial cells (GFAP) in the CA1 region of both NTG and A53T mice treated with a single IP administration of either Veh or 2.5 mg/Kg LPS. (D) Scatter plots and bars (mean±SEM) are the quantitative analysis of the hippocampal normalised percentage GFAP-marked area of the different experimental groups investigated (Two-way ANOVA confirmed a significant interaction *genotype x LPS*: $F_{1,17}=25.15$, $P=0.0001$; * $p<0.05$ and ** $p<0.01$, Tukey's test).

9.2.7 Peripheral LPS does not affect motor behaviour and dopaminergic neurodegeneration in A53T mice

It has been previously reported that the peripheral administration of LPS triggers a progressive dopaminergic neurodegeneration in an α -syn Tg mouse model (Gao *et al.*, 2011). Thus, we have investigated whether the IP administration of 2.5 mg/Kg LPS in either NTG or A53T mice influences their motor behaviour and dopaminergic loss in the SNpc of A53T mice.

As initial approach we measured at functional level the spontaneous motor behaviour of both NTG and A53T mice treated with either Veh or LPS in the open field. As shown in figure 54A and B, A53T mice receiving Veh or LPS (n=7/group) were hyperactive. In fact, the two-way ANOVA found a significant effect of *genotype* for both the parameters considered (Velocity: $F_{1,24}=32.69$; $P<0.00001$. Distance: $F_{1,24}=36.70$; $P<0.0001$). In addition, we showed that the peripheral LPS challenge did not exacerbate A53T spontaneous motor hyperactivity. Consistently, the two-way ANOVA did not find either a significant interaction *genotype x LPS* (Velocity: $F_{1,24}=7.916$; $P=0.096$. Distance: $F_{1,24}=8.047$; $P=0.009$) or a significant effect of *LPS* (Velocity: $F_{1,24}=3.183$; $P=0.087$. Distance: $F_{1,24}=3.202$; $P=0.086$). Of note, hyperactivity in A53T mice has been already reported by other investigators (Graham and Sidhu, 2010; Paumier *et al.*, 2013; Singh *et al.*, 2019), and herein we demonstrate that the peripheral administration of LPS did not worsen it one month after treatment. To further investigate the motor behaviour in our “double-hit” Tg mouse model, we evaluated the mouse gait instability through the Beam-walk test (Figure 54C). As for the spontaneous motor behaviour, the LPS treatment did not influence the number of A53T foot-slips. Indeed, the two-way ANOVA did not find a significant interaction *genotype x LPS 2.5* ($F_{1,24}=1.689$; $P=0.21$) or a significant effect of *LPS 2.5* ($F_{1,24}=2.712$; $P=0.11$). However, we demonstrated that the genetic PD-related background led to a significant increase in the

mean number of A53T mouse foot-slips compared to those of their NTG littermates. In fact, the two-way ANOVA found a significant effect of *genotype* ($F_{1,24}=54.77$; $P<0.0001$).

Of note, despite the lack of a significant difference in motor performances comparing A53T+Veh- and A53T+LPS-treated mice, a trend in their worsening due to the peripheral LPS challenge was detectable.

Since motor impairment in PD is attributable to the selective loss of dopaminergic neurons in the SNpc, we assessed the extent of dopaminergic neurodegeneration in the SNpc investigating the expression of the limiting Th enzyme involved in dopamine synthesis. Particularly, we evaluated whether the trends in the aggravation of motor performances in A53T+LPS-treated animals were due to an LPS-induced loss of dopaminergic neurons. Our analyses revealed both qualitatively and quantitatively (Figure 54D and E) the lack of a significant neuronal loss between A53T+Veh- and A53T+LPS-treated mice.

In conclusion our findings demonstrate that the peripheral LPS treatment does not exacerbate motor behaviour performances of A53T mice. Moreover, our evidence is supported by the lack of LPS-induced dopaminergic neurodegeneration in our “double-hit” Tg model. Of note, our results are consistent with previous data reporting peripheral LPS-induced dopaminergic neurodegeneration in A53T mice not earlier than three months after treatment (Gao *et al.*, 2011).

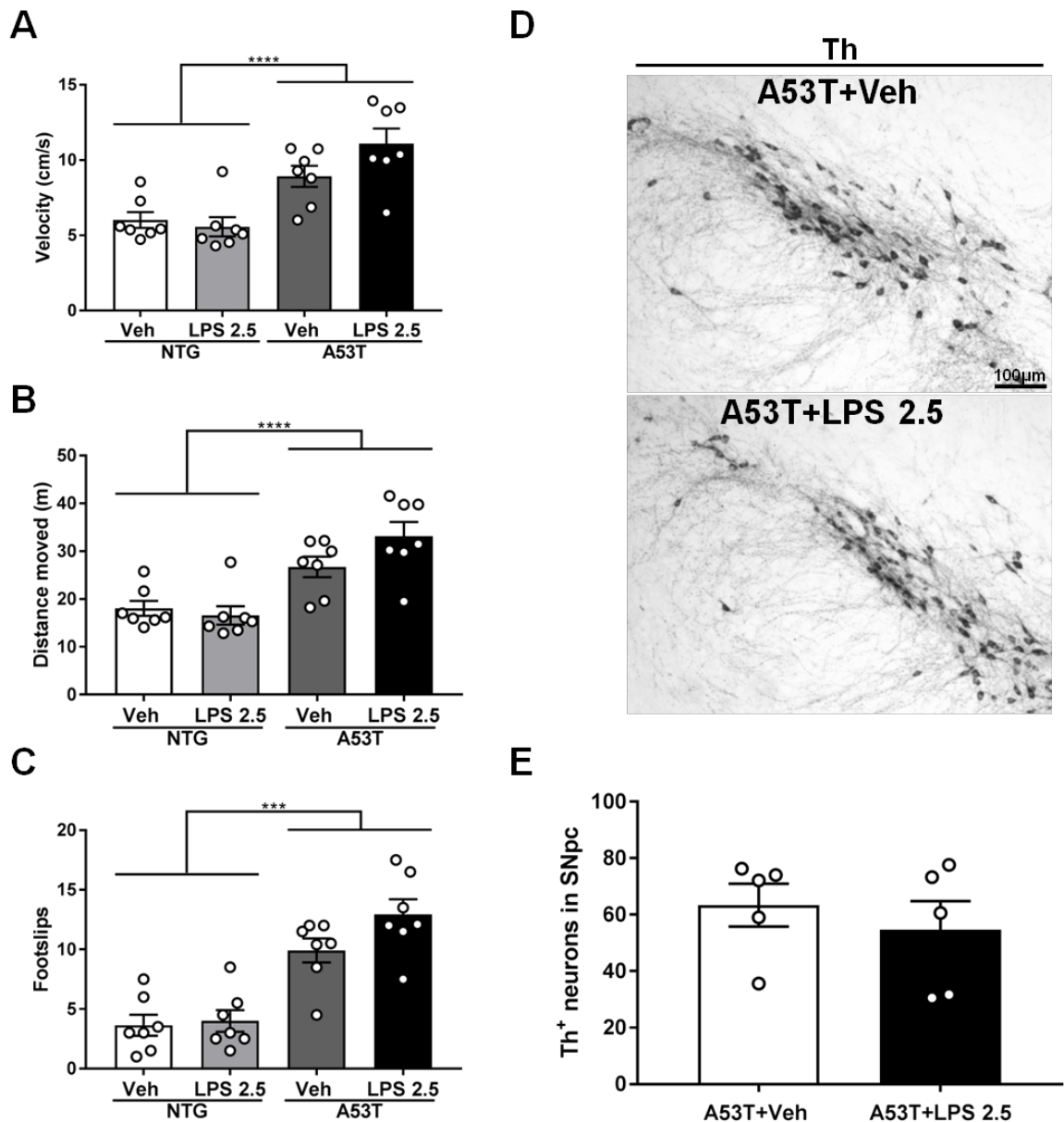


Figure 54. Peripheral administration of 2.5 mg/Kg LPS does not influence motor behaviour of A53T mice and does not trigger dopaminergic neurodegeneration within the SNpc. (A) Scatter plots and bars (mean±SEM) are the total moved distance in the open field of both NTG and A53T mice treated with either Veh or 2.5 mg/Kg LPS (**** $P<0.0001$, Two-way ANOVA). (B) Scatter plots with bars (mean±SEM) are the velocity in the open field of both NTG and A53T mice treated with either Veh or 2.5 mg/Kg LPS (**** $P<0.0001$, Two-way ANOVA). (C) Scatter plots with bars (mean±SEM) are the number of foot-slips in the beam walk test of both NTG and A53T mice treated with either Veh or 2.5 mg/Kg LPS (**** $P<0.01$, Two-way ANOVA). (D) Representative images showing the dopaminergic neurons (Th⁺) in the SNpc of A53T mice treated with a single IP administration of either Veh or 2.5 mg/Kg LPS. (E) Scatter plots with bars (mean±SEM) are the quantitative analysis of SNpc Th⁺ neurons in A53T-treated mice ($t_8=0.51$, $P=0.51$; Student's T-test).

9.3 Discussion

Alpha-synucleinopathies are multi-factorial disorders arising from the interaction between modifiable, and non-modifiable risk factors including genetic susceptibility (Kalia and Lang, 2015; Outeiro *et al.*, 2019). Aimed at elucidating the link between these factors, neuroinflammation has acquired a new light. Accordingly, epidemiological studies reveal that inappropriate management of systemic diseases, turning into an inflammatory response within the CNS, correlates with an increased risk to develop PD (McKenzie *et al.*, 2017). Consistently, experimental data highlight how chronic neuroinflammation, which triggers a deleterious microenvironment, represents a vital link between α -syn pathology and progressive neurodegeneration (Qin *et al.*, 2007; Gao *et al.*, 2011; Wang *et al.*, 2019).

In addition, glial cells interact with α -synOs, mediating their detrimental effects on both memory and neuroinflammation (Kim *et al.*, 2013; Fellner *et al.*, 2013; Kim *et al.*, 2016; La Vitola *et al.*, 2018; La Vitola *et al.*, 2019). Thus, such a condition would promote a neuroinflammatory vicious circle likely favouring and/or aggravating both neuropathological and clinical PD features.

In this chapter we corroborated the theory that inflammation is a driving force for the development of PD or LBD.

In vitro, we proved that non-toxic concentration of α -synOs induced hippocampal neuronal cell death when neurons were pre-exposed to LPS, thus indicating that the presence of an inflammatory milieu increases neuronal susceptibility to α -synOs. To further investigate the influence of LPS pre-conditioning on α -synO effects we have developed an *in vivo* “double-hit” acute mouse model.

We first identified 0.5 μ M as an inactive α -synO concentration which did not affect memory when ICV injected in C57BL/6 naïve mice. In addition, we demonstrated that a single IP administration of 2.5 mg/Kg LPS in C57BL/6 naïve mice led to an immune response within the CNS characterised by persistent activation of glial cells up to one month with no effects

on memory. In contrast, through the “double-hit” mouse model we found that the combination of the two *per se* inactive challenges led to an impairment of both long- and short-term memory. Collectively, our *in vivo* findings are consistent with *in vitro* data and further demonstrate that a peripherally pre-established LPS-induced neuroinflammation influences α -synO harmful effects. Moreover, our findings highlight a synergistic action occurring between neuroinflammation and α -synOs.

Since astroglial and microglial cells are crucial players in tuning synaptic functions and in mediating the α -synO detrimental activities (Bacci *et al.*, 1999; Blank and Printz, 2013; Jang *et al.*, 2011; Hertz and Chen, 2016; La Vitola *et al.*, 2018), we have investigated the extent of hippocampal glial activation in our “double-hit” acute model immediately after the NORT. We found a different response of microglia and astrocytes to the LPS+ α -synOs challenge. While α -synOs potentiate the activation of LPS-primed microglia, which acquired an M1 pro-inflammatory phenotype, astroglial cells appeared atrophic. Such a different response was unexpected and will require further investigation to unveil the mechanisms involved. However, in line with our findings, while microgliosis is widely accepted and reported in PD, contradictory results were obtained for astrocytes which were described as active, inactive or in an atrophic state (McGeer *et al.*, 1988; Chao *et al.*, 2014; Surendranathan *et al.*, 2015; Tong *et al.*, 2015). Thus, although the clear role of astrocytes in PD remains unclear, loss of astrocytic support to neurons might contribute to its development and/or pathogenesis (Sorrentino *et al.*, 2019). Moreover, it is possible to speculate that the acquisition of an M1 pro-inflammatory phenotype of microglial cells together with the astrocytic dysfunction/alteration, likely act in concert to promote neuronal damage, which in turn may underlie the memory impairment in our “double-hit” acute model.

To get more insight on the impact of the peripherally LPS-induced neuroinflammation in a more complex neuropathological context, we developed a “double-hit” model exploiting a

Tg mouse model which carries the A53T PD-related mutation. In agreement with the results obtained in the acute “double-hit” model, we found that A53T mice treated with 2.5 mg/Kg LPS displayed a further impairment in their recognition and spatial memory in association with an increased microgliosis. Of note, in these mice we identified again a reduction in the expression of the astroglial marker GFAP when compared with A53T+Veh mice. In contrast to this deleterious effect at cognitive level, the LPS-treatment did not further impair motor performances in A53T mice. Notably, such an effect was associated with the lack of a significant increased dopaminergic neurodegeneration in the SNpc of A53T mice receiving 2.5 mg/Kg LPS compared to Veh-treated Tg mice.

Taken together our findings demonstrate for the first time that peripherally induced neuroinflammation modulates α -synO action potentiating their detrimental effects. In addition, in a genetic pathologic PD context, the peripheral LPS administration aggravates cognitive deficiencies in A53T mice. In a translational prospect, these results are compelling because, in line with the aforementioned clinical data, clearly indicate how inflammatory events, even from the peripheral compartment, and especially if perpetuating throughout time, represent a concrete risk for the development or exacerbation of PD pathogenesis.

Summary and Final Remarks

Chapter X

10.1 Summary and final remarks

PD and LBD are progressive and devastating neurodegenerative disorders which share common clinical and neuropathological features (Bengoa-Vergniory *et al.*, 2017; Poewe *et al.*, 2017; Jellinger, 2018; Outeiro *et al.*, 2019). Collectively such pathologies represent the second most common neurodegenerative disorders in elderly after AD, and since they frequently lead to institutionalisation, they account for high public health costs (Emre, 2003; Biundo *et al.*, 2016; Hanagasi *et al.*, 2017; Aarsland *et al.*, 2017). To date, PD/LBD are orphans of valuable therapies, and the existing treatments do not affect disease onset/progression being solely symptomatic. Thus, the understanding of the mechanisms underlying their pathogenesis as well as the identification of therapeutic targets aimed at developing new disease modifying therapeutic strategies is a crucial and urgent topic.

As mentioned above, PD and LBD are closely related disorders. Indeed, they share common non-motor clinical features such as cognitive deficits which can evolve in dementia, and common neuropathological hallmarks which include proteinaceous neuronal inclusions known as LBs and LNs (McKeith *et al.*, 2004; Wakabayashi *et al.*, 2013; Aldridge *et al.*, 2018; Sanford, 2018).

Although the first description of PD was provided in 1817 by James Parkinson, the understanding of the underlying mechanisms remained foggy until the identification of different mutations in the gene encoding for α -syn in familial forms of PD and LBD (Polymeropoulos *et al.*, 1997; Krüger *et al.*, 1998; Singleton *et al.*, 2003; Chartier-Harlin *et al.*, 2004; Ibáñez *et al.*, 2004; Zarranz *et al.*, 2004; Proukakis *et al.* 2013; Kiely *et al.* 2013; Lesage *et al.*, 2013; Pasanen *et al.*, 2014), and the first observation that α -syn is the main constituent of both LBs and LNs in post-mortem brain from both PD and DLB patients (Spillantini *et al.*, 1997; Spillantini *et al.*, 1998; Baba *et al.*, 1998). Thus, these findings pinpoint α -syn as a crucial protein involved in the pathogenesis of PD and PD-related disorders.

α -syn is a partially unfolded protein mainly located in the pre-synaptic terminals within the CNS. Its physiological function is still poorly understood. However, it is widely accepted that α -syn is a key protein involved in the neurotransmitter release as well as in the vesicle pool distribution and organisation (Lashuel *et al.*, 2013; Burré *et al.*, 2015; Calo *et al.*, 2016). Although α -syn is a cytoplasmatic protein and LBs and LNs are located within the cells, numerous findings suggest that α -syn can be secreted by human neuronal cells and can be detected in human fluids (*i.e.* plasma and cerebrospinal fluid) (Borghi *et al.* 2000; El-Agnaf *et al.*, 2003; Ohrfelt *et al.*, 2009). These observations raised the interest for the extracellular role of α -syn in the pathogenesis of the so called α -synucleinopathies.

As described above, α -syn is a naturally unfolded protein lacking a well-organised secondary structure, and like other proteins involved in protein-misfolding related neurodegenerative disorders (A β , PrP^C), it is able to self-aggregate. In fact, α -syn undergoes a nucleation process that generates oligomeric species (Maries *et al.*, 2013; Roberts and Brown, 2015). Oligomers are able to grow through further monomer addition and form protofilaments and eventually mature fibrils. Several kinds of oligomers that vary in composition, conformation and toxicity (Danzer *et al.*, 2007; Roberts and Brown, 2015) are generated during this process, and compelling evidence highlights their pathogenetic role in α -synucleinopathies (Winner *et al.*, 2011; Cremades *et al.*, 2012). In addition, it has been reported that stress conditions increase the amount of α -synOs being released (Jang *et al.*, 2010), further supporting the hypothesis that extracellular moieties of α -syn, and particularly extracellular α -synOs, may lead to neuronal damage and contribute to disease pathogenesis. In this regard, it has been demonstrated that α -synOs can impair the LTP, an experimental paradigm aimed at assessing synaptic plasticity, whereas monomers or fibrils of the protein are ineffective (Diogenes *et al.*, 2012. Martin *et al.*, 2012 Ferreira *et al.*, 2017). On the same line, through behavioural *in vivo* experiments, it has been demonstrated that the ICV injection of α -synOs in mice triggers cognitive dysfunction in the fear conditioning paradigm (Martin *et al.*, 2012). All together,

these data support the “oligomeric hypothesis”, originally introduced in the context of AD, and allow researchers to introduce a new definition of α -synucleinopathies as pathologies related to oligomers, and to classify them under the name of “oligomeropathies” (Forloni *et al.*, 2016; Ono, 2017; Ono, 2018).

Based on these findings, the first aim of this PhD thesis was to investigate the α -syn “oligomeric hypothesis” in the context of memory impairment. Of note, our interest in cognitive decline stems from the fact that it is a common PD-related non-motor symptom, and that it contributes to a significant extent to morbidity, worsening life quality of patients (Aarsland *et al.*, 2017; Hanagasi *et al.*, 2017). Moreover, cognitive impairment may develop in dementia, that in PD as well in LBD accounts for high public health cost thus being an urgent issue to cope with (Emre, 2003; Biundo *et al.*, 2016; Aarsland *et al.*, 2017; Hanagasi *et al.*, 2017).

To serve such a purpose we have developed an acute mouse model based on a single ICV injection of different and well-characterised α -syn moieties (monomers, α -synOs and fibrils) in C57BL/6 naïve, and we have assessed their memory performance in the NORT. To be noticed that, although this model represents a simplification of what really happens in the complex context of PD and PD-related disorders, it allows us to punctually dissect the harmful effects of the different α -syn assemblies without any bias due to their co-existence, which for instance occurs in PD Tg mouse models. Through our acute approach we demonstrate that while monomeric and fibrillar α -syn were ineffective in mediating the memory impairment, a single ICV injection of α -synOs was able to cause a memory deficiency in the NORT. Moreover, since pre-treatment with an anti- α -syn antibody completely abrogated the memory deficit in α -synO-treated mice, we can conclude that the memory damage was specifically due to α -syn. Accordingly, our *in vivo* results are supported by *ex vivo* observations that the exposure of coronal brain slices to α -synOs significantly impaired the hippocampal LTP as also previously reported by others (Diogenes *et al.*, 2012;

Martin *et al.*, 2012; Ferreira *et al.*, 2017). To further investigate the α -synO-mediated memory impairment, we assessed whether it was persistent and whether it could be ascribed to macroscopic morphological alterations in the hippocampus or in the expression of representative hippocampal synaptic proteins. Our results demonstrate that α -synO-mediated memory impairment was transient. In fact, mice re-tested in the NORT 12 days after the first α -synO ICV injection performed well in the NORT. Of note, this result is consistent with the absence of significant macroscopic alterations in the different hippocampal subfields as well as with the absence of changes in the expression of both hippocampal synaptophysin and PSD95 at different time points.

Collectively, through our acute mouse model we demonstrate that α -synOs specifically lead to a transient cognitive impairment. Moreover, since the different assemblies of α -syn do not co-exist in our model, it appears as a valuable tool to specifically dissect the mechanisms underlying α -synO-detrimental activities (La Vitola *et al.*, 2018).

As previously described, in pathological and stress conditions neurons increase the amount of α -synOs being secreted in their milieu. Thus, extracellular α -synOs may contribute to the pathogenesis of PD and PD-related disorders by exerting their detrimental action in a non-cell autonomous fashion which involves neighbouring neurons, astrocytes and microglial cells (Marques and Outeiro, 2012). In this regard, many groups started focusing their efforts on the non-cell autonomous actions of α -synOs, which include neuroinflammation and protein-protein interaction.

As extensively described in the introduction (chapter III), several lines of evidence point out the potential role of glial cells in the pathogenesis of PD and PD-related disorders. Astrocytes as well as microglial cells are immune competent cells within the CNS, and they play vital roles for neurons, being crucial effectors in the fine tuning of neuronal activity and synaptic plasticity (Bacci *et al.*, 1999; Blank and Printz, 2013; Hertz and Chen, 2016).

Therefore, perturbation of their function/state might lead to neuronal damage, dysfunction and ultimately death (Morris *et al.*, 2013; Blank and Prinz, 2013; Sorrentino *et al.*, 2019).

Because of a growing body of evidence, the second aim of this PhD thesis was to decipher the role of the neuroinflammatory response in mediating the memory damage triggered by α -synOs. As TLRs are first line receptors involved in eliciting the innate immune response, and α -synOs have been described as one of their endogenous ligands (Kouli *et al.*, 2019), we have investigated whether the memory deficit mediated by α -synOs in our acute mouse model was associated with glial cell activation and was mediated by TLRs. In this regard, based on their controversial role, we have focused our attention mainly on TLR2 and TLR4. Indeed, both TLR2 and TLR4 were reported to mediate astroglial and microglial cell activation upon exposure to different α -syn aggregates (Fellner *et al.*, 2013; Kim *et al.*, 2013; Ramikko *et al.*, 2015; Kim *et al.*, 2016; Kouli *et al.*, 2019), and their expression is altered in PD/LBD patients as well as in PD Tg animal models (Letiembre *et al.*, 2009; Doorn *et al.*, 2014; Drouin-Ouellet *et al.*, 2015; Dzamko *et al.*, 2017; Kim *et al.*, 2018; Zhao *et al.*, 2018). Taking advantage of our acute mouse model, we demonstrate that the single ICV injection of α -synOs (1 μ M/7.5 μ L) triggered a rapid and transient activation of both microglial and astroglial cells in the hippocampus. However, in contrast to the ICV injection of A β Os (1 μ M/7.5 μ L), α -synOs do not lead to an increased expression of the pro-inflammatory mediator IL-1 β . Such a difference is particularly relevant in the context of protein-misfolding related neurodegenerative disorders. As a matter of fact, while oligomeric assemblies of α -syn and A β are recognised as key moieties in the pathogenesis of α -synucleinopathies and AD, respectively, and share common harmful properties, they probably differ in the underlying signalling pathways.

Once demonstrated that α -synOs trigger the activation of glial cells in our acute mouse model, we addressed whether such a neuroinflammatory state was merely a secondary event or represented a key point and a mechanism eliciting the α -synO-mediated memory damage.

As previously reported for A β Os in a comparable acute mouse model (Balducci *et al.*, 2017), we demonstrate that neuroinflammation is a crucial player also in mediating the detrimental effect of oligomeric α -syn on cognitive performance. In fact, through the pre-treatment with two different NSAIDs (Indo and IBF), we show that modulation of inflammatory mediators such as COX1 and 2 completely abrogates α -synO-induced memory deficiency and hippocampal gliosis. Furthermore, in contrast to A β Os (Balducci *et al.*, 2017), α -synO-induced memory impairment is not mediated by TLR4 but is TLR2-dependent (La Vitola *et al.*, 2018). Remarkably, our findings on the involvement of TLR2 are consistent with recent data provided by Kim and co-workers (Kim *et al.*, 2018), and depict TLR2 and neuroinflammation as valuable targets to counteract α -synO detrimental effects.

As mentioned above, α -synOs and A β Os share common toxic properties. Indeed, both α -synOs and A β Os are capable to trigger a memory damage closely related to the induction of hippocampal gliosis (Balducci *et al.*, 2017; La Vitola *et al.*, 2018). In addition, neuroinflammation appears as a key mechanism underlying the detrimental effects of both α -synOs and A β Os on memory (Balducci *et al.*, 2017; La Vitola *et al.*, 2018).

Focusing on common interactors between these different oligomers, it has been recently reported that the PrP^C, initially described with contradictory results as an interactor for A β Os, also represents an interactor and a mediator for α -synOs (Lauren *et al.*, 2009; Balducci *et al.*, 2010; Forloni and Balducci, 2011; Ferreira *et al.*, 2017).

In the context of α -synucleinopathies, it has been found that PrP^C residues 93-109 are crucial to mediate LTP inhibition upon exposure to α -synOs, and α -synOs seem to require PrP^C to activate the Fyn kinase, which in turn leads to NMDAR2B phosphorylation giving rise to LTP inhibition. Moreover, Ferreira and colleagues, demonstrated through immunoprecipitation approaches that α -synOs and PrP^C can interact in lysates from both cell cultures treated with α -synOs and in a PD Tg mouse model overexpressing the WT human α -syn (Ferreira *et al.*, 2017). Based on these data we have further investigated the α -synO-

PrP^C interaction at multiple levels. By means of *Prnp*^{+/+} and *Prnp*^{0/0} neuronal hippocampal cultures, we demonstrate that α -synOs lead to neuronal death in a PrP^C-independent manner. Moreover, through the ICV injection of α -synOs (1 μ M/7.5 μ L) in both *Prnp*^{+/+} and *Prnp*^{0/0} mice, we functionally show that the PrP^C was not required to trigger α -synO-detrimental effects on memory. Besides, in the same model we report that α -synOs caused hippocampal gliosis (La Vitola *et al.*, 2019), thus suggesting that neuroinflammation may represent a non-cell autonomous mechanism eliciting α -synO effects independently of the genetic background considered. In addition to functional data, we also demonstrate at the molecular level that α -synOs do not interact directly with PrP^C (La Vitola *et al.*, 2019), further indicating that α -synOs exert their harmful actions independently of the presence of PrP^C. Our results are in contrast with the findings of Ferreira and colleagues (Ferreira *et al.*, 2017) but, as previously reported for A β Os, both PrP^C-dependent and -independent mechanisms may co-exist. Nevertheless, our findings are also in line with a recent study in A53T mice reporting the absence of Fyn activation and phosphorylation of the NMDARs subunit GluN2B, two downstream outcomes of PrP^C activation (Singh *et al.*, 2019).

Hitherto, our data depict α -synOs as the main culprits eliciting cognitive dysfunctions in mice, and neuroinflammation as a key player in mediating their actions. Thus, although neuroinflammation has been initially considered as a mere secondary event and a consequence of the underlying neurodegenerative process in α -synucleinopathies, we pinpoint neuroinflammation as a non-cell-autonomous mechanism (La Vitola *et al.*, 2018). Our evidence is consistent with previous data, and strongly supports the modulation of neuroinflammatory mediators as valuable strategies to develop new therapies aimed at counteracting α -synOs effects (Kim *et al.*, 2013; Drouin-Ouellet *et al.*, 2015; Kim *et al.*, 2018).

While we depict neuroinflammation as an action mechanism of α -synOs, emerging findings propose neuroinflammation as a potential bridge between non-modifiable (genetic

susceptibility) and modifiable (environment, systemic pathologies) factors co-fostering PD and PD-related disorders (Qin *et al.*, 2007; Gao *et al.*, 2011; Ascherio and Schwarzschild, 2016; McKenzie *et al.*, 2017; Biosa *et al.*, 2018). However, a direct proof demonstrating that inflammation influences α -synO harmful activities or the PD behavioural and neuropathological features is still missing. Hence, the last aim of this PhD thesis was to verify whether a peripherally induced neuroinflammatory state could enhance α -synO effects in our acute mouse model, and whether it influenced the PD phenotype in the more complex context of the A53T Tg PD mouse model.

Taking advantage from a “double-hit” acute mouse model based on the IP injection of the widely used proinflammatory stimulus LPS (2.5mg/Kg) followed one month later by the ICV injection of an ineffective dose of α -synOs (0.5 μ M/7.5 μ L), we demonstrate that the combination of the two *per se* inactive challenges led to an impairment of both long- and short-term memory. Thus, our findings highlight a synergistic action occurring between peripherally LPS-induced neuroinflammation and α -synOs.

As astroglial and microglial cells are immune competent cells within the CNS, and they are crucial in tuning synaptic functions and in mediating the α -synO detrimental activities (Bacci *et al.*, 1999; Blank and Printz, 2013; Jang *et al.*, 2011; Hertz and Chen, 2016; La Vitola *et al.*, 2018), we have investigated the hippocampal glial activation in our newly established “double-hit” acute model immediately after the NORT. Intriguingly, microglial and astroglial cells differentially responded to the double challenge. In fact, while α -synOs potentiated the activation of peripherally LPS-primed microglia, astroglial cells were not activated. Such a different response was unexpected. However, consistent with our findings, while microgliosis is widely accepted and reported in PD and LBD, controversial results were presented for astrocytes which are shown as active, inactive or in an atrophic state (McGeer *et al.*, 1988; Chao *et al.*, 2014; Surendranathan *et al.*, 2015; Tong *et al.*, 2015). Thus, although the role of astrocytes in PD/LBD still remains unclear, loss of astrocytic

support to neurons might contribute synergistically with the acquisition of an M1 pro-inflammatory phenotype of microglial cells towards the memory impairment appearing in our “double-hit” acute model.

To further elucidate and validate the relevance of the peripherally LPS-induced neuroinflammation in PD, we have developed a “double-hit” model exploiting a Tg mouse model which carries the A53T PD-related mutation, and which represents a more complex neuropathological context. In line with the acute “double-hit” model, we found that 2.5 mg/Kg LPS worsened both recognition and spatial memory performance of A53T mice in association with an increased hippocampal microgliosis. Of note, in these mice we show again a reduction in the expression of the astroglial marker GFAP when compared with A53T+Veh mice. In contrast to this deleterious effect at cognitive level, the LPS-treatment did not further aggravate the motor performances and the dopaminergic neurodegeneration in A53T mice. Altogether, we demonstrate for the first time that peripherally induced neuroinflammation modulates α -synO action by potentiating their detrimental effects. In addition, in a genetic PD context, the peripheral LPS administration aggravates cognitive deficiencies in association with a potentiation of microglial cell activation. Intriguingly, while microglial cells are potentiated in their activation state in both our “double-hit” models, astroglial cells are not. Therefore, such a comparable microglial and astroglial outcome in both our models may allow the speculation that in complex contexts, where both peripheral and central events take place, microglial activation and loss of astroglial support to neurons together contribute to the cognitive failure.

In conclusion, the data here reported pinpoint α -synOs as the sole moieties capable of inducing a memory damage in strict association with glial cell activation and independent of the PrP^C in mice (La Vitola *et al.*, 2018; La Vitola *et al.*, 2019). Moreover, although we cannot rule out that extracellular α -synOs act on neurons, we demonstrate that neuroinflammation is not a secondary event but actively participates to the detrimental

effects of oligomers. Of note, since neurons express several inflammatory mediators including TLRs it is possible to speculate that α -synOs exert their effect at multiple levels involving neurons, astroglial and microglial cells at the same time (Dzamko *et al.*, 2017).

On the other hand, our results also propose inflammation as a bridge between genetic susceptibility and environmental factors co-fostering PD/LBD pathogenesis. We indeed reported that *in vitro* the LPS neuronal pre-conditioning enhances α -synO-mediated cell toxicity, and that *in vivo* the peripherally LPS-induced neuroinflammation potentiates α -synO detrimental effects on different memory domains as well as the cognitive deficiencies in A53T mice. These findings are compelling from a translational standpoint as they clearly demonstrate how inflammatory events from the peripheral compartment represent a risk factor for the development and/or the exacerbation of PD pathogenesis. Because α -synucleinopathies are now recognised as multifactorial disorders, our discoveries describe the concomitant action of α -syn aggregates and neuroinflammation, and depict them as valuable targets for developing disease modifying strategies.

10.1.1 Conclusions

Protein misfolding-related neurodegenerative disorders such as PD and LBD are multifactorial pathologies where several factors act in concert leading to neurodegeneration. Aside from the enormous amount of factors involved in PD/LBD pathogenesis, several cellular pathways may be affected and synergistically contribute to the disorder onset and progression (Marques and Outeiro, 2012; Forloni *et al.*, 2016; Bengoa-Vergniory *et al.*, 2017; Wong and Krainc. 2017; Kouli *et al.*, 2019).

The results described here pinpoint for the first time the tight interplay between α -synOs and neuroinflammation in mediating cognitive deficits in both an acute and a transgenic PD/LBD mouse model.

α -synOs have emerged as the main detrimental aggregate in PD/LBD (Winner *et al.*, 2010; Martin *et al.*, 2012 Diogenes *et al.*, 2012). Our data further corroborate previous evidence providing new insights into the crucial role of glial cells. Moreover, we described the novel connection between α -synO-triggered cognitive deficit and non-cell autonomous mechanisms involving glial activation and TLR2 in an acute mouse model, where the detrimental effects at cognitive level are specifically mediated by oligomeric assemblies, and not by the coexistence of larger aggregates.

As mentioned above, PD and LBD are multifactorial pathologies arising from alterations in several pathways (*e.g.* altered neurotransmitter release, reduced neuroplasticity, damage of mitochondria, changes in membrane permeability, increase neuroinflammation, neuronal death, *etc.*). Such a complexity generates controversy in pre-clinical and clinical studies. In fact, while a number of compounds have been shown to ameliorate or block PD/LBD pathology (*e.g.* minocycline, ibuprofen ...) in several animal models, they failed in clinical trials. Therefore, it becomes clear that a therapy based on a molecule affecting solely a single target cannot be beneficial in the complex *scenario* of these pathologies when applied to humans.

As a consequence, it is conceivable to assume that a multitarget approach could be more effective than a single target therapy. Based on the results reported here, a therapeutic approach that may affect at the same time α -syn aggregation as well as neuroinflammation may offer a useful tool to halt PD/LBD progression. In this regard, it has been recently demonstrated that the antimicrobial drug Doxycycline inhibits the aggregation of α -syn, leading to the production of non-toxic off-set α -synOs (Gonzalez-Lizarraga *et al.*, 2017). In addition, Doxycycline has proven effective in drug-induced PD mouse model (Bortolanza *et al.*, 2018), and we have recently demonstrated that the antimicrobial drug administered at sub-antibiotic doses efficiently counteracts memory impairment and neuroinflammation in a transgenic mouse model of AD, and in an acute mouse model based on the ICV injection

of the pro-inflammatory toxin LPS (Balducci *et al.*, 2018). Thus, we are now investigating whether the chronic treatment of A53T mice with Doxycycline is effective in counteracting cognitive and motor deficits as well as neuropathology in our model. In particular, we will focus on the effect of Doxycycline on glial activation, α -syn aggregation and TLR2, which we have demonstrated being crucial in α -synO-induced cognitive damage.

In conclusion, this PhD thesis provides new evidence on the role of α -synOs and neuroinflammation in the pathogenesis of PD/LBD, and puts forward the hypothesis whereby drugs affecting simultaneously these two factors may be valuable therapeutic approaches acting on two discrete elements which are closely connected.

Bibliography

- Aarsland, D., Creese, B., Politis, M., Chaudhuri, K.R., Ffytche, D.H., Weintraub, D., and Ballard, C. (2017). Cognitive decline in Parkinson disease. *Nat Rev Neurol* 13, 217–231.
- Aguzzi, A., Barres, B.A., and Bennett, M.L. (2013). Microglia: Scapegoat, Saboteur, or Something Else? *Science* 339, 156–161.
- Aldridge, G.M., Birnschein, A., Denburg, N.L., and Narayanan, N.S. (2018). Parkinson's Disease Dementia and Dementia with Lewy Bodies Have Similar Neuropsychological Profiles. *Front. Neurol.* 9, 123.
- Alvarez-Erviti, L., Couch, Y., Richardson, J., Cooper, J.M., and Wood, M.J.A. (2011). Alpha-synuclein release by neurons activates the inflammatory response in a microglial cell line. *Neurosci. Res.* 69, 337–342.
- Andersen, P., and Lømo, T. (1966). Mode of activation of hippocampal pyramidal cells by excitatory synapses on dendrites. *Exp Brain Res* 2, 247–260.
- Anderson, J.P., Walker, D.E., Goldstein, J.M., de Laat, R., Banducci, K., Caccavello, R.J., Barbour, R., Huang, J., Kling, K., Lee, M., *et al.* (2006). Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J. Biol. Chem.* 281, 29739–29752.
- Arroyo, D.S., Soria, J.A., Gaviglio, E.A., Rodriguez-Galan, M.C., and Iribarren, P. (2011). Toll-like receptors are key players in neurodegeneration. *International Immunopharmacology* 11, 1415–1421.
- Ascherio, A., and Schwarzschild, M.A. (2016). The epidemiology of Parkinson's disease: risk factors and prevention. *Lancet Neurol* 15, 1257–1272.
- Baba, M., Nakajo, S., Tu, P.H., Tomita, T., Nakaya, K., Lee, V.M., Trojanowski, J.Q., and Iwatsubo, T. (1998). Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. *Am. J. Pathol.* 152, 879–884.
- Bacci, A., Verderio, C., Pravettoni, E., and Matteoli, M. (1999). The role of glial cells in synaptic function. *Phil. Trans. R. Soc. Lond. B* 354, 403–409.
- Bailey, C.H., and Kandel, E.R. (1993). Structural changes accompanying memory storage. *Annu. Rev. Physiol.* 55, 397–426.
- Balducci, C., and Forloni, G. (2014). In Vivo Application of beta Amyloid Oligomers: A Simple Tool to Evaluate Mechanisms of Action and New Therapeutic Approaches. *CPD* 20, 2491–2505.
- Balducci, C., Beeg, M., Stravalaci, M., Bastone, A., Scip, A., Biasini, E., Tapella, L., Colombo, L., Manzoni, C., Borsello, T., *et al.* (2010). Synthetic amyloid-beta oligomers impair long-term memory independently of cellular prion protein. *Proc Natl Acad Sci U S A* 107, 2295–2300.
- Balducci, C., Frasca, A., Zotti, M., La Vitola, P., Mhillaj, E., Grigoli, E., Iacobellis, M., Grandi, F., Messa, M., Colombo, L., *et al.* (2016). Toll-like receptor 4-dependent glial cell activation mediates the impairment in memory establishment induced by β -amyloid oligomers in an acute mouse model of Alzheimer's disease. *Brain Behav. Immun.*

- Balducci, C., Frasca, A., Zotti, M., La Vitola, P., Mhillaj, E., Grigoli, E., Iacobellis, M., Grandi, F., Messa, M., Colombo, L., *et al.* (2017). Toll-like receptor 4-dependent glial cell activation mediates the impairment in memory establishment induced by β -amyloid oligomers in an acute mouse model of Alzheimer's disease. *Brain Behav. Immun.* *60*, 188–197.
- Balducci, C., Santamaria, G., La Vitola, P., Brandi, E., Grandi, F., Viscomi, A.R., Beeg, M., Gobbi, M., Salmona, M., Ottonello, S., *et al.* (2018). Doxycycline counteracts neuroinflammation restoring memory in Alzheimer's disease mouse models. *Neurobiol. Aging* *70*, 128–139.
- Baltaci, S.B., Mogulkoc, R., and Baltaci, A.K. (2019). Molecular Mechanisms of Early and Late LTP. *Neurochem. Res.* *44*, 281–296.
- Bartels, T., Choi, J.G., and Selkoe, D.J. (2011). α -Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. *Nature* *477*, 107–110.
- Beier, E.E., Neal, M., Alam, G., Edler, M., Wu, L.-J., and Richardson, J.R. (2017). Alternative microglial activation is associated with cessation of progressive dopamine neuron loss in mice systemically administered lipopolysaccharide. *Neurobiology of Disease* *108*, 115–127.
- Bellucci, A., Zaltieri, M., Navarria, L., Grigoletto, J., Missale, C., and Spano, P. (2012). From α -synuclein to synaptic dysfunctions: new insights into the pathophysiology of Parkinson's disease. *Brain Res.* *1476*, 183–202.
- Bendor, J., Logan, T., and Edwards, R.H. (2013). The Function of α -Synuclein. *Neuron* *79*.
- Bengoa-Vergniory, N., Roberts, R.F., Wade-Martins, R., and Alegre-Abarrategui, J. (2017). Alpha-synuclein oligomers: a new hope. *Acta Neuropathol.* *134*, 819–838.
- Benilova, I., Karran, E., and De Strooper, B. The toxic A β oligomer and Alzheimer's disease: an emperor in need of clothes. *Nat Neurosci* *15*, 349–357.
- Béraud, D., Twomey, M., Bloom, B., Mittereder, A., Ton, V., Neitzke, K., Chasovskikh, S., Mhyre, T.R., and Maguire-Zeiss, K.A. (2011). α -Synuclein Alters Toll-Like Receptor Expression. *Front. Neurosci.* *5*.
- Bergman, H., Wichmann, T., and DeLong, M.R. (1990). Reversal of experimental parkinsonism by lesions of the subthalamic nucleus. *Science* *249*, 1436–1438.
- Betzer, C., Movius, A.J., Shi, M., Gai, W.-P., Zhang, J., and Jensen, P.H. (2015). Identification of synaptosomal proteins binding to monomeric and oligomeric α -synuclein. *PLoS ONE* *10*, e0116473.
- Bhat, S., Acharya, U.R., Hagiwara, Y., Dadmehr, N., and Adeli, H. (2018). Parkinson's disease: Cause factors, measurable indicators, and early diagnosis. *Comput. Biol. Med.* *102*, 234–241.
- Billingsley, K.J., Bandres-Ciga, S., Saez-Atienzar, S., and Singleton, A.B. (2018). Genetic risk factors in Parkinson's disease. *Cell Tissue Res* *373*, 9–20.

- Bisaz, R., Travaglia, A., and Alberini, C.M. (2014). The neurobiological bases of memory formation: from physiological conditions to psychopathology. *Psychopathology* 47, 347–356.
- Biundo, R., Weis, L., and Antonini, A. (2016). Cognitive decline in Parkinson's disease: the complex picture. *NPJ Parkinsons Dis* 2, 16018.
- Blank, T., and Prinz, M. Microglia as modulators of cognition and neuropsychiatric disorders. *Glia* 61, 62–70.
- Blank, T., and Prinz, M. (2013). Microglia as modulators of cognition and neuropsychiatric disorders. *Glia* 61, 62–70.
- Bliss, T.V., and Gardner-Medwin, A.R. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the unanaesthetised rabbit following stimulation of the perforant path. *J. Physiol. (Lond.)* 232, 357–374.
- Blum-Degen, D., Müller, T., Kuhn, W., Gerlach, M., Przuntek, H., and Riederer, P. (1995). Interleukin-1 β and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. *Neuroscience Letters* 202, 17–20.
- Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C.J., Squitieri, F., Ibanez, P., Joosse, M., *et al.* (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 299, 256–259.
- Borghi, R., Marchese, R., Negro, A., Marinelli, L., Forloni, G., Zaccheo, D., Abbruzzese, G., and Tabaton, M. (2000). Full length alpha-synuclein is present in cerebrospinal fluid from Parkinson's disease and normal subjects. *Neurosci. Lett.* 287, 65–67.
- Bortolanza M., Nascimento GC., Socias SB., Ploper D., Chehín RN, Raisman-Vozari R., Del-Bel E. (2018). Tetracycline repurposing in neurodegeneration: focus on Parkinson's disease. *J Neural Transm (Vienna)*. 2018 Oct;125(10):1403-1415. doi: 10.1007/s00702-018-1913-1
- Bourdenx, M., Bezard, E., and Dehay, B. (2014). Lysosomes and α -synuclein form a dangerous duet leading to neuronal cell death. *Front Neuroanat* 8, 83.
- Braak, H., and Braak, E. (2000). Pathoanatomy of Parkinson's disease. *J. Neurol.* 247 Suppl 2, II3-10.
- Braak, H., Del Tredici, K., Rüb, U., de Vos, R.A.I., Jansen Steur, E.N.H., and Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol. Aging* 24, 197–211.
- Bridi, J.C., and Hirth, F. (2018). Mechanisms of α -Synuclein Induced Synaptopathy in Parkinson's Disease. *Front Neurosci* 12, 80.
- Brodacki, B., Staszewski, J., Toczyłowska, B., Kozłowska, E., Drela, N., Chalimoniuk, M., and Stepień, A. (2008). Serum interleukin (IL-2, IL-10, IL-6, IL-4), TNF α , and INF γ concentrations are elevated in patients with atypical and idiopathic parkinsonism. *Neuroscience Letters* 441, 158–162.

- Buchman, V.L., Hunter, H.J., Pinõn, L.G., Thompson, J., Privalova, E.M., Ninkina, N.N., and Davies, A.M. (1998). Persyn, a member of the synuclein family, has a distinct pattern of expression in the developing nervous system. *J. Neurosci.* *18*, 9335–9341.
- Bueler, H., Aguzzi, A., Sailer, A., Greiner, R.A., Autenried, P., Aguet, M., and Weissmann, C. (1993). Mice devoid of PrP are resistant to scrapie. *Cell* *73*, 1339–1347.
- Buell, A.K., Galvagnion, C., Gaspar, R., Sparr, E., Vendruscolo, M., Knowles, T.P.J., Linse, S., and Dobson, C.M. (2014). Solution conditions determine the relative importance of nucleation and growth processes in α -synuclein aggregation. *Proc. Natl. Acad. Sci. U.S.A.* *111*, 7671–7676.
- Burré, J., Sharma, M., Tsetsenis, T., Buchman, V., Etherton, M.R., and Südhof, T.C. (2010). Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science* *329*, 1663–1667.
- Burré, J., Sharma, M., and Südhof, T.C. (2015). Definition of a molecular pathway mediating α -synuclein neurotoxicity. *J. Neurosci.* *35*, 5221–5232.
- Burré, J., Sharma, M., and Südhof, T.C. (2018). Cell Biology and Pathophysiology of α -Synuclein. *Cold Spring Harb Perspect Med* *8*.
- Cai, W., Feng, D., Schwarzschild, M.A., McLean, P.J., and Chen, X. (2018). Bimolecular Fluorescence Complementation of Alpha-synuclein Demonstrates its Oligomerisation with Dopaminergic Phenotype in Mice. *EBioMedicine* *29*, 13–22.
- Calabrese, V., Santoro, A., Monti, D., Crupi, R., Di Paola, R., Latteri, S., Cuzzocrea, S., Zappia, M., Giordano, J., Calabrese, E.J., *et al.* (2018). Aging and Parkinson's Disease: Inflammaging, neuroinflammation and biological remodelling as key factors in pathogenesis. *Free Radic. Biol. Med.* *115*, 80–91.
- Caldinelli, L., Albani, D., and Pollegioni, L. (2013). One single method to produce native and Tat-fused recombinant human α -synuclein in Escherichia coli. *BMC Biotechnol* *13*, 32.
- Calo, L., Wegrzynowicz, M., Santivañez-Perez, J., and Grazia Spillantini, M. (2016). Synaptic failure and α -synuclein. *Mov. Disord.* *31*, 169–177.
- Camina, E., and Güell, F. (2017). The Neuroanatomical, Neurophysiological and Psychological Basis of Memory: Current Models and Their Origins. *Front Pharmacol* *8*, 438.
- Campbell, B.C., Li, Q.X., Culvenor, J.G., Jäkälä, P., Cappai, R., Beyreuther, K., Masters, C.L., and McLean, C.A. (2000). Accumulation of insoluble alpha-synuclein in dementia with Lewy bodies. *Neurobiol. Dis.* *7*, 192–200.
- Carlsson, A. (2001). A half-century of neurotransmitter research: impact on neurology and psychiatry. *Nobel lecture. Biosci. Rep.* *21*, 691–710.
- Carlsson, A., Lindqvist, M., Magnusson, T., and Waldeck, B. (1958). On the presence of 3-hydroxytyramine in brain. *Science* *127*, 471.
- Charcot J.M. (1875). *Leçons aur les maladies du système nerveux*. Vol 1. Delhaye et Cie, Paris.

- Chao, Y., Wong, S.-C., and King Tan, E. (2014). Evidence of Inflammatory System Involvement in Parkinson's Disease. *BioMed Research International* 2014, 308654.
- Chartier, S., and Duyckaerts, C. (2018). Is Lewy pathology in the human nervous system chiefly an indicator of neuronal protection or of toxicity? *Cell Tissue Res.* 373, 149–160.
- Chartier-Harlin, M.-C., Kachergus, J., Roumier, C., Mouroux, V., Douay, X., Lincoln, S., Levecque, C., Larvor, L., Andrieux, J., Hulihan, M., *et al.* (2004). Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. *Lancet* 364, 1167–1169.
- Chaudhary, H., Iyer, A., Subramaniam, V., and Claessens, M.M.A.E. (2016). α -Synuclein Oligomers Stabilize Pre-Existing Defects in Supported Bilayers and Propagate Membrane Damage in a Fractal-Like Pattern. *Langmuir* 32, 11827–11836.
- Cherry, J.D., Olschowka, J.A., and O'Banion, M. (2014). Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *J Neuroinflammation* 11, 98.
- Chiesa, R., Piccardo, P., Ghetti, B., and Harris, D.A. (1998). Neurological illness in transgenic mice expressing a prion protein with an insertional mutation. *Neuron* 21, 1339–1351.
- Choi, B.-K., Choi, M.-G., Kim, J.-Y., Yang, Y., Lai, Y., Kweon, D.-H., Lee, N.K., and Shin, Y.-K. (2013). Large α -synuclein oligomers inhibit neuronal SNARE-mediated vesicle docking. *Proc. Natl. Acad. Sci. U.S.A.* 110, 4087–4092.
- Choi, D.-Y., Liu, M., Hunter, R.L., Cass, W.A., Pandya, J.D., Sullivan, P.G., Shin, E.-J., Kim, H.-C., Gash, D.M., and Bing, G. (2009). Striatal neuroinflammation promotes Parkinsonism in rats. *PLoS ONE* 4, e5482.
- Citri, A., and Malenka, R.C. (2008). Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology* 33, 18–41.
- Civiero, L., Cogo, S., Biossa, A., and Greggio, E. (2018). The role of LRRK2 in cytoskeletal dynamics. *Biochem. Soc. Trans.* 46, 1653–1663.
- Clough, Z., Jeyapaul, P., Zotova, E., and Holmes, C. (2013). Proinflammatory Cytokines and the Clinical Features of Dementia With Lewy Bodies. *Alzheimer Disease and Associated Disorders* 29.
- Conway, K.A., Lee, S.J., Rochet, J.C., Ding, T.T., Williamson, R.E., and Lansbury, P.T. (2000). Acceleration of oligomerisation, not fibrillisation, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc. Natl. Acad. Sci. U.S.A.* 97, 571–576.
- Cuenca, L., Gil-Martinez, A.L., Cano-Fernandez, L., Sanchez-Rodrigo, C., Estrada, C., Fernandez-Villalba, E., and Herrero, M.T. (2019). Parkinson's disease: a short story of 200 years. *Histol. Histopathol.* 34, 573–591.
- Członkowska, A., Kohutnicka, M., Kurkowska-Jastrzębska, I., and Członkowski, A. (1996). Microglial Reaction in MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) Induced Parkinson's Disease Mice Model. *Neurodegeneration* 5, 137–143.

- Dahlström, A., and Fuxe, K. (1964). Localisation of monoamines in the lower brain stem. *Experientia* 20, 398–399.
- Danyu, L., Yanran, L., Xiuna, J., Ying, C., Sudan, P., Tianen, Z., Zhifen, Z., Dezhi, Z., Kaixun, H., Yingyu, X., *et al.* (2019). α -Synuclein induced mitochondrial dysfunction via cytochrome c oxidase subunit 2 in SH-SY5Y cells. *Experimental Cell Research* 378, 57–65.
- Danzer, K.M., Haasen, D., Karow, A.R., Moussaud, S., Habeck, M., Giese, A., Kretschmar, H., Hengerer, B., and Kostka, M. (2007). Different species of alpha-synuclein oligomers induce calcium influx and seeding. *J. Neurosci.* 27, 9220–9232.
- Danzer, K.M., Kranich, L.R., Ruf, W.P., Cagsal-Getkin, O., Winslow, A.R., Zhu, L., Vanderburg, C.R., and McLean, P.J. (2012). Exosomal cell-to-cell transmission of alpha synuclein oligomers. *Mol Neurodegener* 7, 42.
- Deleersnijder, A., Gerard, M., Debyser, Z., and Baekelandt, V. (2013). The remarkable conformational plasticity of alpha-synuclein: blessing or curse? *Trends Mol Med* 19, 368–377.
- Dickson, D.W. (2018). Neuropathology of Parkinson disease. *Parkinsonism Relat. Disord.* 46 Suppl 1, S30–S33.
- Diogenes, M.J., Dias, R.B., Rombo, D.M., Vicente Miranda, H., Maiolino, F., Guerreiro, P., Näsström, T., Franquelim, H.G., Oliveira, L.M.A., Castanho, M.A.R.B., *et al.* (2012). Extracellular alpha-synuclein oligomers modulate synaptic transmission and impair LTP via NMDA-receptor activation. *J. Neurosci.* 32, 11750–11762.
- di Domenico, A., Carola, G., Calatayud, C., Pons-Espinal, M., Muñoz, J.P., Richaud-Patin, Y., Fernandez-Carasa, I., Gut, M., Faella, A., Parameswaran, J., *et al.* (2019). Patient-Specific iPSC-Derived Astrocytes Contribute to Non-Cell-Autonomous Neurodegeneration in Parkinson's Disease. *Stem Cell Reports* 12, 213–229.
- Donzis, E.J., and Tronson, N.C. (2014). Modulation of learning and memory by cytokines: signalling mechanisms and long term consequences. *Neurobiol Learn Mem* 115, 68–77.
- Doorn, K.J., Moors, T., Drukarch, B., van de Berg, W.D., Lucassen, P.J., and van Dam, A.-M. (2014). Microglial phenotypes and toll-like receptor 2 in the substantia nigra and hippocampus of incidental Lewy body disease cases and Parkinson's disease patients. *Acta Neuropathol Commun* 2, 90.
- Dorsey, E.R., Constantinescu, R., Thompson, J.P., Biglan, K.M., Holloway, R.G., Kieburtz, K., Marshall, F.J., Ravina, B.M., Schifitto, G., Siderowf, A., *et al.* (2007). Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. *Neurology* 68, 384–386.
- Driver, J.A., Logroscino, G., Gaziano, J.M., and Kurth, T. (2009). Incidence and remaining lifetime risk of Parkinson disease in advanced age. *Neurology* 72, 432–438.
- Drouin-Ouellet, J., St-Amour, I., Saint-Pierre, M., Lamontagne-Proulx, J., Kriz, J., Barker, R.A., and Cicchetti, F. (2015). Toll-Like Receptor Expression in the Blood and Brain of Patients and a Mouse Model of Parkinson's Disease. *International Journal of Neuropsychopharmacology* 18, pyu103–pyu103.

- Dzamko, N., Geczy, C., and M Halliday, G. (2014). Inflammation is genetically implicated in Parkinson's disease. *Neuroscience* 302.
- Dzamko, N., Geczy, C., and Halliday, G. (2015). Inflammation is genetically implicated in Parkinson's disease. *Neuroscience* 302, 89–102.
- Dzamko, N., Gysbers, A., Perera, G., Bahar, A., Shankar, A., Gao, J., Fu, Y., and Halliday, G.M. (2017). Toll-like receptor 2 is increased in neurons in Parkinson's disease brain and may contribute to alpha-synuclein pathology. *Acta Neuropathol* 133, 303–319.
- Ehringer, H., and Hornykiewicz, O. (1960). [Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behaviour in diseases of the extrapyramidal system]. *Klin. Wochenschr.* 38, 1236–1239.
- Eidson, L.N., Kannarkat, G.T., Barnum, C.J., Chang, J., Chung, J., Caspell-Garcia, C., Taylor, P., Mollenhauer, B., Schlossmacher, M.G., Ereshefsky, L., *et al.* (2017). Candidate inflammatory biomarkers display unique relationships with alpha-synuclein and correlate with measures of disease severity in subjects with Parkinson's disease. *Journal of Neuroinflammation* 14, 164.
- El-Agnaf, O.M.A., Salem, S.A., Paleologou, K.E., Cooper, L.J., Fullwood, N.J., Gibson, M.J., Curran, M.D., Court, J.A., Mann, D.M.A., Ikeda, S., *et al.* (2003). Alpha-synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma. *FASEB J.* 17, 1945–1947.
- Eliezer, D., Kutluay, E., Bussell, R., and Browne, G. (2001). Conformational properties of alpha-synuclein in its free and lipid-associated states. *J. Mol. Biol.* 307, 1061–1073.
- Emmanouilidou, E., Elenis, D., Papasilekas, T., Stranjalis, G., Gerozissis, K., Ioannou, P.C., and Vekrellis, K. (2011). Assessment of α -synuclein secretion in mouse and human brain parenchyma. *PLoS ONE* 6, e22225.
- Emre, M. (2003). Dementia associated with Parkinson's disease. *Lancet Neurol* 2, 229–237.
- Fahn, S. (2015). The medical treatment of Parkinson disease from James Parkinson to George Cotzias. *Mov. Disord.* 30, 4–18.
- Fahn, S. (2018). The 200-year journey of Parkinson disease: Reflecting on the past and looking towards the future. *Parkinsonism Relat. Disord.* 46 Suppl 1, S1–S5.
- Farrer, M.J. (2006). Genetics of Parkinson disease: paradigm shifts and future prospects. *Nat. Rev. Genet.* 7, 306–318.
- Fellner, L., and Stefanova, N. (2013). The role of glia in α -synucleinopathies. *Mol. Neurobiol.* 47, 575–586.
- Fellner, L., Jellinger, K.A., Wenning, G.K., and Stefanova, N. (2011). Glial dysfunction in the pathogenesis of α -synucleinopathies: emerging concepts. *Acta Neuropathol.* 121, 675–693.
- Fellner, L., Irschick, R., Schanda, K., Reindl, M., Klimaschewski, L., Poewe, W., Wenning, G.K., and Stefanova, N. (2013). Toll-like receptor 4 is required for α -synuclein dependent activation of microglia and astroglia. *Glia* 61, 349–360.

- Fernandez-Perez, E.J., Peters, C., and Aguayo, L.G. (2016). Membrane Damage Induced by Amyloid Beta and a Potential Link with Neuroinflammation. *Curr. Pharm. Des.* 22, 1295–1304.
- Ferrari, C., Pott Godoy, C., Tarelli, R., Chertoff, M., Depino, A., and Pitossi, F. (2006). Progressive neurodegeneration and motor disabilities induced by chronic expression of IL-1 β in the substantia nigra. *Neurobiology of Disease* 24, 183–193.
- Ferreira, D.G., Temido-Ferreira, M., Vicente Miranda, H., Batalha, V.L., Coelho, J.E., Szegő, É.M., Marques-Morgado, I., Vaz, S.H., Rhee, J.S., Schmitz, M., *et al.* (2017). α -synuclein interacts with PrPC to induce cognitive impairment through mGluR5 and NMDAR2B. *Nat. Neurosci.* 20, 1569–1579.
- Flagmeier, P., Meisl, G., Vendruscolo, M., Knowles, T.P.J., Dobson, C.M., Buell, A.K., and Galvagnion, C. (2016). Mutations associated with familial Parkinson's disease alter the initiation and amplification steps of α -synuclein aggregation. *Proc. Natl. Acad. Sci. U.S.A.* 113, 10328–10333.
- Forloni, G., Artuso, V., La Vitola, P., and Balducci, C. (2016). Oligomeropathies and pathogenesis of Alzheimer and Parkinson's diseases. *Mov Disord* 31, 771–781.
- Foroud, T., Uniacke, S.K., Liu, L., Pankratz, N., Rudolph, A., Halter, C., Shults, C., Marder, K., Conneally, P.M., Nichols, W.C., *et al.* (2003). Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. *Neurology* 60, 796–801.
- Frigerio, R., Fujishiro, H., Ahn, T.-B., Josephs, K.A., Maraganore, D.M., DelleDonne, A., Parisi, J.E., Klos, K.J., Boeve, B.F., Dickson, D.W., *et al.* (2011). Incidental Lewy body disease: do some cases represent a preclinical stage of dementia with Lewy bodies? *Neurobiol. Aging* 32, 857–863.
- Funk, N., Wieghofer, P., Grimm, S., Schaefer, R., Bühring, H.-J., Gasser, T., and Biskup, S. (2013). Characterisation of peripheral hematopoietic stem cells and monocytes in Parkinson's disease: Parkinson's Disease and Innate Immunity. *Mov Disord.* 28, 392–395.
- Galasko, D. (2017). Lewy Body Disorders. *Neurol Clin* 35, 325–338.
- Gallegos, S., Pacheco, C., Peters, C., Opazo, C.M., and Aguayo, L.G. (2015). Features of alpha-synuclein that could explain the progression and irreversibility of Parkinson's disease. *Front Neurosci* 9, 59.
- Galvan, A., Devergnas, A., and Wichmann, T. (2015). Alterations in neuronal activity in basal ganglia-thalamocortical circuits in the parkinsonian state. *Front Neuroanat* 9.
- Gandhi, S., and Plun-Favreau, H. (2017). Mutations and mechanism: how PINK1 may contribute to risk of sporadic Parkinson's disease. *Brain* 140, 2–5.
- Gao, H.-M., Kotzbauer, P.T., Uryu, K., Leight, S., Trojanowski, J.Q., and Lee, V.M.-Y. (2008). Neuroinflammation and oxidation/nitration of alpha-synuclein linked to dopaminergic neurodegeneration. *J. Neurosci.* 28, 7687–7698.
- Gao, H.-M., Zhang, F., Zhou, H., Kam, W., Wilson, B., and Hong, J.-S. (2011). Neuroinflammation and α -synuclein dysfunction potentiate each other, driving chronic

progression of neurodegeneration in a mouse model of Parkinson's disease. *Environ. Health Perspect.* 119, 807–814.

Gelders, G., Baekelandt, V., and Van der Perren, A. (2018). Linking Neuroinflammation and Neurodegeneration in Parkinson's Disease. *Journal of Immunology Research* 2018, 1–12.

George, J.M. (2002). The synucleins. *Genome Biol.* 3, REVIEWS3002.

Giasson, B.I., Murray, I.V., Trojanowski, J.Q., and Lee, V.M. (2001). A hydrophobic stretch of 12 amino acid residues in the middle of alpha-synuclein is essential for filament assembly. *J. Biol. Chem.* 276, 2380–2386.

Glass, C.K., Saijo, K., Winner, B., Marchetto, M.C.N., and Gage, F.H. (2010). Mechanisms Underlying Inflammation in Neurodegeneration. *Cell* 140, 918–934.

Gloeckner, C.J., Schumacher, A., Boldt, K., and Ueffing, M. (2009). The Parkinson disease-associated protein kinase LRRK2 exhibits MAPKKK activity and phosphorylates MKK3/6 and MKK4/7, in vitro. *J. Neurochem.* 109, 959–968.

Goedert, M., Jakes, R., and Spillantini, M.G. (2017). The Synucleinopathies: Twenty Years On. *J Parkinsons Dis* 7, S51–S69.

González-Lizárraga F., Socías SB., Ávila CL., Torres-Bugeau CM., Barbosa LR., Binolfi A., Sepúlveda-Díaz JE, Del-Bel E., Fernandez CO., Papy-Garcia D., Itri R., Raisman-Vozari R., Chehín RN.. (2019). Repurposing doxycycline for synucleinopathies: remodelling of α -synuclein oligomers towards non-toxic parallel beta-sheet structured species. *Sci Rep.* 2017 Feb 3;7:41755. doi: 10.1038/srep41755.

Goetz, C.G. (2011). The history of Parkinson's disease: early clinical descriptions and neurological therapies. *Cold Spring Harb Perspect Med* 1, a008862.

Graham, D.R., and Sidhu, A. (2010a). Mice expressing the A53T mutant form of human alpha-synuclein exhibit hyperactivity and reduced anxiety-like behaviour. *J. Neurosci. Res.* NA-NA.

Graham, D.R., and Sidhu, A. (2010b). Mice expressing the A53T mutant form of human alpha-synuclein exhibit hyperactivity and reduced anxiety-like behaviour. *J. Neurosci. Res.* 88, 1777–1783.

Greenbaum, E.A., Graves, C.L., Mishizen-Eberz, A.J., Lupoli, M.A., Lynch, D.R., Englander, S.W., Axelsen, P.H., and Giasson, B.I. (2005). The E46K mutation in alpha-synuclein increases amyloid fibril formation. *J. Biol. Chem.* 280, 7800–7807.

Gupta, V., Garg, R.K., and Khattri, S. (2016). Levels of IL-8 and TNF- α decrease in Parkinson's disease. *Neurological Research* 38, 98–102.

Hall, S., Janelidze, S., Surova, Y., Widner, H., Zetterberg, H., and Hansson, O. (2018). Cerebrospinal fluid concentrations of inflammatory markers in Parkinson's disease and atypical parkinsonian disorders. *Sci Rep* 8, 13276.

Halliday, G., Lees, A., and Stern, M. (2011). Milestones in Parkinson's disease--clinical and pathologic features. *Mov. Disord.* 26, 1015–1021.

- Hanagasi, H.A., Tufekcioglu, Z., and Emre, M. (2017). Dementia in Parkinson's disease. *J. Neurol. Sci.* 374, 26–31.
- Hansson, O., Hall, S., Ohrfelt, A., Zetterberg, H., Blennow, K., Minthon, L., Nägga, K., Londos, E., Varghese, S., Majbour, N.K., *et al.* (2014). Levels of cerebrospinal fluid α -synuclein oligomers are increased in Parkinson's disease with dementia and dementia with Lewy bodies compared to Alzheimer's disease. *Alzheimers Res Ther* 6, 25.
- Harms, A.S., Thome, A.D., Yan, Z., Schonhoff, A.M., Williams, G.P., Li, X., Liu, Y., Qin, H., Benveniste, E.N., and Standaert, D.G. (2018). Peripheral monocyte entry is required for alpha-Synuclein induced inflammation and Neurodegeneration in a model of Parkinson disease. *Experimental Neurology* 300, 179–187.
- Hedrich, K., Eskelson, C., Wilmot, B., Marder, K., Harris, J., Garrels, J., Meija-Santana, H., Vieregge, P., Jacobs, H., Bressman, S.B., *et al.* (2004). Distribution, type, and origin of Parkin mutations: review and case studies. *Mov. Disord.* 19, 1146–1157.
- Heneka, M.T., Kummer, M.P., and Latz, E. (2014). Innate immune activation in neurodegenerative disease. *Nature Reviews Immunology* 14, 463–477.
- Hepp, D.H., Vergoossen, D.L.E., Huisman, E., Lemstra, A.W., Netherlands Brain Bank, Berendse, H.W., Rozemuller, A.J., Foncke, E.M.J., and van de Berg, W.D.J. (2016). Distribution and Load of Amyloid- β Pathology in Parkinson Disease and Dementia with Lewy Bodies. *J. Neuropathol. Exp. Neurol.* 75, 936–945.
- Hernandez, D.G., Reed, X., and Singleton, A.B. (2016). Genetics in Parkinson disease: Mendelian versus non-Mendelian inheritance. *J. Neurochem.* 139 Suppl 1, 59–74.
- Hertz, L., and Chen, Y. (2016). Editorial: All 3 Types of Glial Cells Are Important for Memory Formation. *Front. Integr. Neurosci.* 10.
- Heusinkveld, L.E., Hacker, M.L., Turchan, M., Davis, T.L., and Charles, D. (2018). Impact of Tremor on Patients With Early Stage Parkinson's Disease. *Front Neurol* 9, 628.
- Hirsch, E.C., Vyas, S., and Hunot, S. (2012). Neuroinflammation in Parkinson's disease. *Parkinsonism & Related Disorders* 18, S210–S212.
- Hirsch, E. C. & Hunot, S. (2009). Neuroinflammation in Parkinson's disease: a target for neuroprotection? *Lancet Neurol.* 8, 382–397. *Lancet Neurology* 8, 382–397.
- Huang, Z., Xu, Z., Wu, Y., and Zhou, Y. (2011). Determining nuclear localisation of alpha-synuclein in mouse brains. *Neuroscience* 199, 318–332.
- Hüls, S., Högen, T., Vassallo, N., Danzer, K.M., Hengerer, B., Giese, A., and Herms, J. (2011). AMPA-receptor-mediated excitatory synaptic transmission is enhanced by iron-induced α -synuclein oligomers. *J. Neurochem.* 117, 868–878.
- Ibáñez, P., Bonnet, A.-M., Débarges, B., Lohmann, E., Tison, F., Pollak, P., Agid, Y., Dürr, A., and Brice, A. (2004). Causal relation between alpha-synuclein gene duplication and familial Parkinson's disease. *Lancet* 364, 1169–1171.
- Ingelsson, M. (2016). Alpha-Synuclein Oligomers-Neurotoxic Molecules in Parkinson's Disease and Other Lewy Body Disorders. *Front Neurosci* 10, 408.

- Irizarry, M.C., Kim, T.W., McNamara, M., Tanzi, R.E., George, J.M., Clayton, D.F., and Hyman, B.T. (1996). Characterisation of the precursor protein of the non-A beta component of senile plaques (NACP) in the human central nervous system. *J. Neuropathol. Exp. Neurol.* 55, 889–895.
- Iwai, A., Masliah, E., Yoshimoto, M., Ge, N., Flanagan, L., de Silva, H.A., Kittel, A., and Saitoh, T. (1995). The precursor protein of non-A beta component of Alzheimer's disease amyloid is a presynaptic protein of the central nervous system. *Neuron* 14, 467–475.
- Jain, N., Bhasne, K., Hemaswasthi, M., and Mukhopadhyay, S. (2013). Structural and dynamical insights into the membrane-bound α -synuclein. *PLoS ONE* 8, e83752.
- Jakes, R., Spillantini, M.G., and Goedert, M. (1994). Identification of two distinct synucleins from human brain. *FEBS Lett.* 345, 27–32.
- Jana, M., Palencia, C.A., and Pahan, K. (2008). Fibrillar Amyloid- β Peptides Activate Microglia via TLR2: Implications for Alzheimer's Disease. *J Immunol* 181, 7254–7262.
- Jang, A., Lee, H.-J., Suk, J.-E., Jung, J.-W., Kim, K.-P., and Lee, S.-J. (2010). Non-classical exocytosis of alpha-synuclein is sensitive to folding states and promoted under stress conditions. *J. Neurochem.* 113, 1263–1274.
- Jankovic, J. (2008). Parkinson's disease: clinical features and diagnosis. *J. Neurol. Neurosurg. Psychiatry* 79, 368–376.
- Jellinger, K.A. (2014). Neuropathology of Parkinson's Disease. In *Inflammation in Parkinson's Disease: Scientific and Clinical Aspects*, M. Thomas, ed. (Cham: Springer International Publishing), pp. 25–74.
- Jellinger, K.A. (2018). Dementia with Lewy bodies and Parkinson's disease-dementia: current concepts and controversies. *J Neural Transm (Vienna)* 125, 615–650.
- Ji, H., Liu, Y.E., Jia, T., Wang, M., Liu, J., Xiao, G., Joseph, B.K., Rosen, C., and Shi, Y.E. (1997). Identification of a breast cancer-specific gene, BCSG1, by direct differential cDNA sequencing. *Cancer Res.* 57, 759–764.
- Joe, E.-H., Choi, D.-J., An, J., Eun, J.-H., Jou, I., and Park, S. (2018). Astrocytes, Microglia, and Parkinson's Disease. *Exp Neurobiol* 27, 77.
- Jung, Y.-J., and Chung, W.-S. (2018). Phagocytic Roles of Glial Cells in Healthy and Diseased Brains. *Biomolecules & Therapeutics* 26, 350–357.
- Kalia, L.V., and Lang, A.E. (2015). Parkinson's disease. *Lancet* 386, 896–912.
- Kempuraj, D., Thangavel, R., Selvakumar, G.P., Zaheer, S., Ahmed, M.E., Raikwar, S.P., Zahoor, H., Saeed, D., Natteru, P.A., Iyer, S., *et al.* (2017). Brain and Peripheral Atypical Inflammatory Mediators Potentiate Neuroinflammation and Neurodegeneration. *Front. Cell. Neurosci.* 11, 216.
- Kiely, A.P., Asi, Y.T., Kara, E., Limousin, P., Ling, H., Lewis, P., Proukakis, C., Quinn, N., Lees, A.J., Hardy, J., *et al.* (2013). α -Synucleinopathy associated with G51D SNCA mutation: a link between Parkinson's disease and multiple system atrophy? *Acta Neuropathol.* 125, 753–769.

- Kim, C., Ho, D.-H., Suk, J.-E., You, S., Michael, S., Kang, J., Joong Lee, S., Masliah, E., Hwang, D., Lee, H.-J., *et al.* (2013). Neuron-released oligomeric α -synuclein is an endogenous agonist of TLR2 for paracrine activation of microglia. *Nat Commun* 4, 1562.
- Kim, C., Lee, H.-J., Masliah, E., and Lee, S.-J. (2016). Non-cell-autonomous Neurotoxicity of α -synuclein Through Microglial Toll-like Receptor 2. *Exp Neurobiol* 25, 113–119.
- Kim, C., Spencer, B., Rockenstein, E., Yamakado, H., Mante, M., Adame, A., Fields, J.A., Masliah, D., Iba, M., Lee, H.-J., *et al.* (2018). Immunotherapy targeting toll-like receptor 2 alleviates neurodegeneration in models of synucleinopathy by modulating α -synuclein transmission and neuroinflammation. *Mol Neurodegeneration* 13, 43.
- Kontopoulos, E., Parvin, J.D., and Feany, M.B. (2006). Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. *Hum. Mol. Genet.* 15, 3012–3023.
- Kouli, A., Horne, C.B., and Williams-Gray, C.H. (2019). Toll-like receptors and their therapeutic potential in Parkinson's disease and α -synucleinopathies. *Brain, Behaviour, and Immunity* S0889159119300820.
- Kramer, M.L., and Schulz-Schaeffer, W.J. (2007). Presynaptic alpha-synuclein aggregates, not Lewy bodies, cause neurodegeneration in dementia with Lewy bodies. *J. Neurosci.* 27, 1405–1410.
- Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Epplen, J.T., Schöls, L., and Riess, O. (1998). Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat. Genet.* 18, 106–108.
- Kuzuhara, S., Mori, H., Izumiyama, N., Yoshimura, M., and Ihara, Y. (1988). Lewy bodies are ubiquitinated. A light and electron microscopic immunocytochemical study. *Acta Neuropathol.* 75, 345–353.
- La Vitola, P., Balducci, C., Cerovic, M., Santamaria, G., Brandi, E., Grandi, F., Caldinelli, L., Colombo, L., Morgese, M.G., Trabace, L., *et al.* (2018a). Alpha-synuclein oligomers impair memory through glial cell activation and via Toll-like receptor 2. *Brain, Behaviour, and Immunity* 69, 591–602.
- La Vitola, P., Balducci, C., Cerovic, M., Santamaria, G., Brandi, E., Grandi, F., Caldinelli, L., Colombo, L., Morgese, M.G., Trabace, L., *et al.* (2018b). Alpha-synuclein oligomers impair memory through glial cell activation and via Toll-like receptor 2. *Brain Behav. Immun.*
- La Vitola, P., Beeg, M., Balducci, C., Santamaria, G., Restelli, E., Colombo, L., Caldinelli, L., Pollegioni, L., Gobbi, M., Chiesa, R., *et al.* (2019). Cellular prion protein neither binds to alpha-synuclein oligomers nor mediates their detrimental effects. *Brain* 142, 249–254.
- Lang, A.E., and Lozano, A.M. (1998). Parkinson's disease. First of two parts. *N. Engl. J. Med.* 339, 1044–1053.
- Larson, M.E., Sherman, M.A., Greimel, S., Kuskowski, M., Schneider, J.A., Bennett, D.A., and Lesné, S.E. (2012). Soluble α -synuclein is a novel modulator of Alzheimer's disease pathophysiology. *J. Neurosci.* 32, 10253–10266.

- Lashuel, H.A., Overk, C.R., Oueslati, A., and Masliah, E. (2013). The many faces of α -synuclein: from structure and toxicity to therapeutic target. *Nat. Rev. Neurosci.* *14*, 38–48.
- Lauren, J., Gimbel, D.A., Nygaard, H.B., Gilbert, J.W., and Strittmatter, S.M. (2009). Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. *Nature* *457*, 1128–1132.
- Lavedan, C. (1998). The Synuclein Family. *Genome Res.* *8*, 871–880.
- Lecours, C., Bordeleau, M., Cantin, L., Parent, M., Paolo, T.D., and Tremblay, M.-È. (2018). Microglial Implication in Parkinson's Disease: Loss of Beneficial Physiological Roles or Gain of Inflammatory Functions? *Front. Cell. Neurosci.* *12*, 282.
- Lee, H.M., and Koh, S.-B. (2015). Many Faces of Parkinson's Disease: Non-Motor Symptoms of Parkinson's Disease. *J Mov Disord* *8*, 92–97.
- Lee, H.-J., Khoshaghideh, F., Patel, S., and Lee, S.-J. (2004). Clearance of alpha-synuclein oligomeric intermediates via the lysosomal degradation pathway. *J. Neurosci.* *24*, 1888–1896.
- Lee, H.-J., Patel, S., and Lee, S.-J. (2005). Intravesicular localisation and exocytosis of alpha-synuclein and its aggregates. *J. Neurosci.* *25*, 6016–6024.
- Lee, H.-J., Bae, E.-J., and Lee, S.-J. (2014). Extracellular α -synuclein-a novel and crucial factor in Lewy body diseases. *Nat Rev Neurol* *10*, 92–98.
- Lee, S.-J., Jeon, H., and Kandror, K.V. (2008). Alpha-synuclein is localised in a subpopulation of rat brain synaptic vesicles. *Acta Neurobiol Exp (Wars)* *68*, 509–515.
- Lemkau, L.R., Comellas, G., Kloepper, K.D., Woods, W.S., George, J.M., and Rienstra, C.M. (2012). Mutant protein A30P α -synuclein adopts wild-type fibril structure, despite slower fibrillation kinetics. *J. Biol. Chem.* *287*, 11526–11532.
- Lesage, S., Dürr, A., and Brice, A. (2006). LRRK2, gène majeur de la maladie de Parkinson dans les pays du Maghreb. *ms* *22*, 470–471.
- Lesage, S., Magali, P., Lohmann, E., Lacomblez, L., Teive, H., Janin, S., Cousin, P.-Y., Dürr, A., Brice, A., and French Parkinson Disease Genetics Study Group (2007). Deletion of the parkin and PACRG gene promoter in early-onset parkinsonism. *Hum. Mutat.* *28*, 27–32.
- Lesage, S., Condroyer, C., Lannuzel, A., Lohmann, E., Troiano, A., Tison, F., Damier, P., Thobois, S., Ouvrard-Hernandez, A.-M., Rivaud-Péchoux, S., *et al.* (2009). Molecular analyses of the LRRK2 gene in European and North African autosomal dominant Parkinson's disease. *J. Med. Genet.* *46*, 458–464.
- Lesage, S., Anheim, M., Letournel, F., Bousset, L., Honoré, A., Rozas, N., Pieri, L., Madiona, K., Dürr, A., Melki, R., *et al.* (2013). G51D α -synuclein mutation causes a novel parkinsonian-pyramidal syndrome. *Ann. Neurol.* *73*, 459–471.
- Letiembre, M., Liu, Y., Walter, S., Hao, W., Pfander, T., Wrede, A., Schulz-Schaeffer, W., and Fassbender, K. (2009). Screening of innate immune receptors in neurodegenerative diseases: A similar pattern. *Neurobiology of Aging* *30*, 759–768.

- Li, J.-Q., Tan, L., and Yu, J.-T. (2014). The role of the LRRK2 gene in Parkinsonism. *Mol Neurodegener* 9, 47.
- Li, J.-Y., Henning Jensen, P., and Dahlström, A. (2002). Differential localisation of alpha-, beta- and gamma-synucleins in the rat CNS. *Neuroscience* 113, 463–478.
- Lim, S., Chun, Y., Lee, J.S., and Lee, S.-J. (2016). Neuroinflammation in Synucleinopathies. *Brain Pathol.* 26, 404–409.
- Lim, S., Kim, H.-J., Kim, D.-K., and Lee, S.-J. (2018). Non-cell-autonomous actions of α -synuclein: Implications in glial synucleinopathies. *Prog. Neurobiol.* 169, 158–171.
- Lim, Y., Kehm, V.M., Lee, E.B., Soper, J.H., Li, C., Trojanowski, J.Q., and Lee, V.M.-Y. (2011). α -Syn suppression reverses synaptic and memory defects in a mouse model of dementia with Lewy bodies. *J. Neurosci.* 31, 10076–10087.
- Lindersson, E., Beedholm, R., Højrup, P., Moos, T., Gai, W., Hendil, K.B., and Jensen, P.H. (2004). Proteasomal inhibition by alpha-synuclein filaments and oligomers. *J. Biol. Chem.* 279, 12924–12934.
- Lücking, C.B., Dürr, A., Bonifati, V., Vaughan, J., De Michele, G., Gasser, T., Harhangi, B.S., Meco, G., Denèfle, P., Wood, N.W., *et al.* (2000). Association between early-onset Parkinson's disease and mutations in the parkin gene. *N. Engl. J. Med.* 342, 1560–1567.
- Mackenzie, I.R. (2000). Activated microglia in dementia with Lewy bodies. *Neurology* 55, 132–134.
- MacLeod, D., Dowman, J., Hammond, R., Leete, T., Inoue, K., and Abeliovich, A. (2006). The familial Parkinsonism gene LRRK2 regulates neurite process morphology. *Neuron* 52, 587–593.
- Mahad, D., Callahan, M.K., Williams, K.A., Ubogu, E.E., Kivisäkk, P., Tucky, B., Kidd, G., Kingsbury, G.A., Chang, A., Fox, R.J., *et al.* (2006). Modulating CCR2 and CCL2 at the blood–brain barrier: relevance for multiple sclerosis pathogenesis. *Brain* 129, 212–223.
- Majbour, N.K., Vaikath, N.N., van Dijk, K.D., Ardah, M.T., Varghese, S., Vesterager, L.B., Montezinho, L.P., Poole, S., Safieh-Garabedian, B., Tokuda, T., *et al.* (2016). Oligomeric and phosphorylated alpha-synuclein as potential CSF biomarkers for Parkinson's disease. *Molecular Neurodegeneration* 11, 7.
- Mandel, S., Grünblatt, E., and Youdim, M. (2000). cDNA microarray to study gene expression of dopaminergic neurodegeneration and neuroprotection in MPTP and 6-hydroxydopamine models: implications for idiopathic Parkinson's disease. In *Advances in Research on Neurodegeneration*, P. Riederer, D.B. Calne, R. Horowski, Y. Mizuno, C.W. Olanow, W. Poewe, and M.B.H. Youdim, eds. (Springer Vienna), pp. 117–124.
- Maroteaux, L., Campanelli, J.T., and Scheller, R.H. (1988). Synuclein: a neuron-specific protein localised to the nucleus and presynaptic nerve terminal. *J. Neurosci.* 8, 2804–2815.
- Marques, O., and Outeiro, T.F. (2012). Alpha-synuclein: from secretion to dysfunction and death. *Cell Death Dis* 3, e350.

- Martin, Z.S., Neugebauer, V., Dineley, K.T., Kaye, R., Zhang, W., Reese, L.C., and Taglialetela, G. (2012). α -Synuclein oligomers oppose long-term potentiation and impair memory through a calcineurin-dependent mechanism: relevance to human synucleopathic diseases. *J. Neurochem.* 120, 440–452.
- Marui, W., Iseki, E., Nakai, T., Miura, S., Kato, M., Ueda, K., and Kosaka, K. (2002). Progression and staging of Lewy pathology in brains from patients with dementia with Lewy bodies. *J. Neurol. Sci.* 195, 153–159.
- McGeer, P.L., and McGeer, E.G. (2008). Glial reactions in Parkinson's disease. *Mov. Disord.* 23, 474–483.
- McGeer, P.L., Itagaki, S., Boyes, B.E., and McGeer, E.G. (1988). Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. *Neurology* 38, 1285–1291.
- McKeith, I., Mintzer, J., Aarsland, D., Burn, D., Chiu, H., Cohen-Mansfield, J., Dickson, D., Dubois, B., Duda, J.E., Feldman, H., *et al.* (2004). Dementia with Lewy bodies. *Lancet Neurol* 3, 19–28.
- McKeith, I.G., Boeve, B.F., Dickson, D.W., Halliday, G., Taylor, J.-P., Weintraub, D., Aarsland, D., Galvin, J., Attems, J., Ballard, C.G., *et al.* (2017). Diagnosis and management of dementia with Lewy bodies: Fourth consensus report of the DLB Consortium. *Neurology* 89, 88–100.
- McKenzie, J.A., Spielman, L.J., Pointer, C.B., Lowry, J.R., Bajwa, E., Lee, C.W., and Klegeris, A. (2017). Neuroinflammation as a Common Mechanism Associated with the Modifiable Risk Factors for Alzheimer's and Parkinson's Diseases. *Curr Aging Sci* 10, 158–176.
- McKimmie, C.S., Johnson, N., Fooks, A.R., and Fazakerley, J.K. (2005). Viruses selectively upregulate Toll-like receptors in the central nervous system. *Biochemical and Biophysical Research Communications* 336, 925–933.
- Menzies, F.M., Yeniseetti, S.C., and Min, K.-T. (2005). Roles of Drosophila DJ-1 in survival of dopaminergic neurons and oxidative stress. *Curr. Biol.* 15, 1578–1582.
- Meyers, R. (1942). The modification of alternating tremors, rigidity and destination by surgery of the basal ganglia. *Res. Publ. Assoc. Res. Nerv. Ment. Dis.* 21, 602–665
- Milber, J.M., Noorigian, J.V., Morley, J.F., Petrovitch, H., White, L., Ross, G.W., and Duda, J.E. (2012). Lewy pathology is not the first sign of degeneration in vulnerable neurons in Parkinson disease. *Neurology* 79, 2307–2314.
- Miller, S., and Mayford, M. (1999). Cellular and molecular mechanisms of memory: the LTP connection. *Current Opinion in Genetics & Development* 9, 333–337.
- Mogi, M., Harada, M., Riederer, P., Narabayashi, H., Fujita, K., and Nagatsu, T. (1994). Tumor necrosis factor- α (TNF- α) increases both in the brain and in the cerebrospinal fluid from parkinsonian patients. *Neuroscience Letters* 165, 208–210.
- Mollenhauer, B., Trautmann, E., Otte, B., Ng, J., Spreer, A., Lange, P., Sixel-Döring, F., Hakimi, M., Vonsattel, J.-P., Nussbaum, R., *et al.* (2012). α -Synuclein in human

cerebrospinal fluid is principally derived from neurons of the central nervous system. *J Neural Transm (Vienna)* 119, 739–746.

Mori, H., Hattori, N., and Mizuno, Y. (2003). Genotype-phenotype correlation: familial Parkinson disease. *Neuropathology* 23, 90–94.

Morris, G.P., Clark, I.A., Zinn, R., and Vissel, B. (2013). Microglia: a new frontier for synaptic plasticity, learning and memory, and neurodegenerative disease research. *Neurobiol Learn Mem* 105, 40–53.

Mortiboys, H., Johansen, K.K., Aasly, J.O., and Bandmann, O. (2010). Mitochondrial impairment in patients with Parkinson disease with the G2019S mutation in LRRK2. *Neurology* 75, 2017–2020.

Mucke, L., and Selkoe, D.J. (2012). Neurotoxicity of Amyloid β -Protein: Synaptic and Network Dysfunction. *Cold Spring Harbor Perspectives in Medicine* 2, a006338–a006338.

Mueller, T., Blum-Degen, D., Przuntek, H., and Kuhn, W. (1998). Interleukin-6 levels in cerebrospinal fluid inversely correlate to severity of Parkinson's disease. *Acta Neurologica Scandinavica* 98, 142–144.

Narhi, L., Wood, S.J., Steavenson, S., Jiang, Y., Wu, G.M., Anafi, D., Kaufman, S.A., Martin, F., Sitney, K., Denis, P., *et al.* (1999). Both familial Parkinson's disease mutations accelerate alpha-synuclein aggregation. *J. Biol. Chem.* 274, 9843–9846.

Nemani, V.M., Lu, W., Berge, V., Nakamura, K., Onoa, B., Lee, M.K., Chaudhry, F.A., Nicoll, R.A., and Edwards, R.H. (2010). Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis. *Neuron* 65, 66–79.

NINDS NET-PD Investigators. (2008). A pilot clinical trial of creatine and minocycline in early Parkinson disease: 18-month results. *Clin Neuropharmacol* 31, 141–50.

Okun, E., Griffioen, K.J., Lathia, J.D., Tang, S.-C., Mattson, M.P., and Arumugam, T.V. (2009). Toll-like receptors in neurodegeneration. *Brain Research Reviews* 59, 278–292.

Olanow, C.W., Perl, D.P., DeMartino, G.N., and McNaught, K.S.P. (2004). Lewy-body formation is an aggresome-related process: a hypothesis. *Lancet Neurol* 3, 496–503.

Ono, K. (2017). The Oligomer Hypothesis in α -Synucleinopathy. *Neurochem. Res.* 42, 3362–3371.

Ono, K. (2018). Alzheimer's disease as oligomeropathy. *Neurochemistry International* 119, 57–70.

Orenstein, S.J., Kuo, S.-H., Tasset, I., Arias, E., Koga, H., Fernandez-Carasa, I., Cortes, E., Honig, L.S., Dauer, W., Consiglio, A., *et al.* (2013). Interplay of LRRK2 with chaperone-mediated autophagy. *Nat. Neurosci.* 16, 394–406.

Ouchi, Y., Yoshikawa, E., Sekine, Y., Futatsubashi, M., Kanno, T., Ogosu, T., and Torizuka, T. (2005). Microglial activation and dopamine terminal loss in early Parkinson's disease. *Ann Neurol.* 57, 168–175.

- Outeiro, T.F., Koss, D.J., Erskine, D., Walker, L., Kurzawa-Akanbi, M., Burn, D., Donaghy, P., Morris, C., Taylor, J.-P., Thomas, A., *et al.* (2019). Dementia with Lewy bodies: an update and outlook. *Mol Neurodegener* 14, 5.
- Overk, C.R., and Masliah, E. (2014). Pathogenesis of synaptic degeneration in Alzheimer's disease and Lewy body disease. *Biochem. Pharmacol.* 88, 508–516.
- Pankratz, N., Pauciulo, M.W., Elsaesser, V.E., Marek, D.K., Halter, C.A., Wojcieszek, J., Rudolph, A., Shults, C.W., Foroud, T., Nichols, W.C., *et al.* (2006). Mutations in DJ-1 are rare in familial Parkinson disease. *Neurosci. Lett.* 408, 209–213.
- Parisiadou, L., Xie, C., Cho, H.J., Lin, X., Gu, X.-L., Long, C.-X., Lobbstaël, E., Baekelandt, V., Taymans, J.-M., Sun, L., *et al.* (2009). Phosphorylation of ezrin/radixin/moesin proteins by LRRK2 promotes the rearrangement of actin cytoskeleton in neuronal morphogenesis. *J. Neurosci.* 29, 13971–13980.
- Park, K.W., Kim, H.S., Cheon, S.-M., Cha, J.-K., Kim, S.-H., and Kim, J.W. (2011). Dementia with Lewy Bodies versus Alzheimer's Disease and Parkinson's Disease Dementia: A Comparison of Cognitive Profiles. *J Clin Neurol* 7, 19–24.
- Parkkinen, L., Kauppinen, T., Pirttilä, T., Autere, J.M., and Alafuzoff, I. (2005). Alpha-synuclein pathology does not predict extrapyramidal symptoms or dementia. *Ann. Neurol.* 57, 82–91.
- Parkkinen, L., Pirttilä, T., and Alafuzoff, I. (2008). Applicability of current staging/categorisation of alpha-synuclein pathology and their clinical relevance. *Acta Neuropathol.* 115, 399–407.
- Pasanen, P., Myllykangas, L., Siitonen, M., Raunio, A., Kaakkola, S., Lyytinen, J., Tienari, P.J., Pöyhönen, M., and Paetau, A. (2014). Novel α -synuclein mutation A53E associated with atypical multiple system atrophy and Parkinson's disease-type pathology. *Neurobiol. Aging* 35, 2180.e1-5.
- Paumier, K.L., Sukoff Rizzo, S.J., Berger, Z., Chen, Y., Gonzales, C., Kaftan, E., Li, L., Lotarski, S., Monaghan, M., Shen, W., *et al.* (2013). Behavioural Characterisation of A53T Mice Reveals Early and Late Stage Deficits Related to Parkinson's Disease. *PLoS ONE* 8, e70274.
- Pickrell, A.M., and Youle, R.J. (2015). The roles of PINK1, parkin, and mitochondrial fidelity in Parkinson's disease. *Neuron* 85, 257–273.
- Pischiutta, F., Micotti, E., Hay, J.R., Marongiu, I., Sammali, E., Tolomeo, D., Vegliante, G., Stocchetti, N., Forloni, G., De Simoni, M.-G., *et al.* (2018). Single severe traumatic brain injury produces progressive pathology with ongoing contralateral white matter damage one year after injury. *Experimental Neurology* 300, 167–178.
- Plotegher, N., Gratton, E., and Bubacco, L. (2014). Number and Brightness analysis of alpha-synuclein oligomerisation and the associated mitochondrial morphology alterations in live cells. *Biochim. Biophys. Acta* 1840, 2014–2024.
- Poewe, W. (2008). Non-motor symptoms in Parkinson's disease. *Eur. J. Neurol.* 15 Suppl 1, 14–20.

- Poewe, W., Seppi, K., Tanner, C.M., Halliday, G.M., Brundin, P., Volkmann, J., Schrag, A.-E., and Lang, A.E. (2017). Parkinson disease. *Nat Rev Dis Primers* 3, 17013.
- Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., *et al.* (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276, 2045–2047.
- Pringsheim, T., Jette, N., Frolkis, A., and Steeves, T.D.L. (2014). The prevalence of Parkinson's disease: a systematic review and meta-analysis. *Mov. Disord.* 29, 1583–1590.
- Prots, I., Veber, V., Brey, S., Campioni, S., Buder, K., Riek, R., Böhm, K.J., and Winner, B. (2013). α -Synuclein oligomers impair neuronal microtubule-kinesin interplay. *J. Biol. Chem.* 288, 21742–21754.
- Proukakis, C., Dudzik, C.G., Brier, T., MacKay, D.S., Cooper, J.M., Millhauser, G.L., Houlden, H., and Schapira, A.H. (2013). A novel α -synuclein missense mutation in Parkinson disease. *Neurology* 80, 1062–1064.
- Przedborski, S. (2017). The two-century journey of Parkinson disease research. *Nat. Rev. Neurosci.* 18, 251–259.
- Purro, S.A., Nicoll, A.J., and Collinge, J. (2018). Prion Protein as a Toxic Acceptor of Amyloid- β Oligomers. *Biological Psychiatry* 83, 358–368.
- Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J.-S., Knapp, D.J., and Crews, F.T. (2007). Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. *Glia* 55, 453–462.
- Rannikko, E.H., Weber, S.S., and Kahle, P.J. (2015). Exogenous α -synuclein induces toll-like receptor 4 dependent inflammatory responses in astrocytes. *BMC Neurosci* 16, 57.
- Reale, M., Iarlori, C., Thomas, A., Gambi, D., Perfetti, B., Di Nicola, M., and Onofri, M. (2009). Peripheral cytokines profile in Parkinson's disease. *Brain, Behaviour, and Immunity* 23, 55–63.
- Restelli, E., Fioriti, L., Mantovani, S., Airaghi, S., Forloni, G., and Chiesa, R. (2010). Cell Type-Specific Neuroprotective Activity of Untranslocated Prion Protein. *PLoS ONE* 5, e13725.
- Richwine, A.F., Sparkman, N.L., Dilger, R.N., Buchanan, J.B., and Johnson, R.W. (2009). Cognitive deficits in interleukin-10-deficient mice after peripheral injection of lipopolysaccharide. *Brain Behav. Immun.* 23, 794–802.
- Roberts, H.L., and Brown, D.R. (2015). Seeking a mechanism for the toxicity of oligomeric α -synuclein. *Biomolecules* 5, 282–305.
- Rosadini, C.V., and Kagan, J.C. (2017). Early innate immune responses to bacterial LPS. *Current Opinion in Immunology* 44, 14–19.
- Rutherford, N.J., Moore, B.D., Golde, T.E., and Giasson, B.I. (2014). Divergent effects of the H50Q and G51D SNCA mutations on the aggregation of α -synuclein. *J. Neurochem.* 131, 859–867.

- Sahin, C., Lorenzen, N., Lemminger, L., Christiansen, G., Möller, I.M., Vesterager, L.B., Pedersen, L.Ø., Fog, K., Kallunki, P., and Otzen, D.E. (2017). Antibodies against the C-terminus of α -synuclein modulate its fibrillation. *Biophys. Chem.* **220**, 34–41.
- Samuel, F., Flavin, W.P., Iqbal, S., Pacelli, C., Sri Renganathan, S.D., Trudeau, L.-E., Campbell, E.M., Fraser, P.E., and Tandon, A. (2016). Effects of Serine 129 Phosphorylation on α -Synuclein Aggregation, Membrane Association, and Internalisation. *J. Biol. Chem.* **291**, 4374–4385.
- Sanford, A.M. (2018). Lewy Body Dementia. *Clin. Geriatr. Med.* **34**, 603–615.
- Savica, R., Grossardt, B.R., Bower, J.H., Boeve, B.F., Ahlskog, J.E., and Rocca, W.A. (2013). Incidence of dementia with Lewy bodies and Parkinson disease dementia. *JAMA Neurol* **70**, 1396–1402.
- Schapira, A.H.V., Chaudhuri, K.R., and Jenner, P. (2017). Non-motor features of Parkinson disease. *Nat. Rev. Neurosci.* **18**, 435–450.
- Schrag, A., Horsfall, L., Walters, K., Noyce, A., and Petersen, I. (2015). Prediagnostic presentations of Parkinson's disease in primary care: a case-control study. *Lancet Neurol* **14**, 57–64.
- Scott, D.A., Tabarean, I., Tang, Y., Cartier, A., Masliah, E., and Roy, S. (2010). A pathologic cascade leading to synaptic dysfunction in alpha-synuclein-induced neurodegeneration. *J. Neurosci.* **30**, 8083–8095.
- Scoville, W.B., and Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *J. Neurol. Neurosurg. Psychiatry* **20**, 11–21.
- Silva, A.J., Kogan, J.H., Frankland, P.W., and Kida, S. (1998). CREB and memory. *Annu. Rev. Neurosci.* **21**, 127–148.
- Singh, B., Covelo, A., Martell-Martínez, H., Nanclares, C., Sherman, M.A., Okematti, E., Meints, J., Teravskis, P.J., Gallardo, C., Savonenko, A.V., *et al.* (2019). Tau is required for progressive synaptic and memory deficits in a transgenic mouse model of α -synucleinopathy. *Acta Neuropathol.*
- Singleton, A.B., Farrer, M., Johnson, J., Singleton, A., Hague, S., Kachergus, J., Hulihan, M., Peuralinna, T., Dutra, A., Nussbaum, R., *et al.* (2003). alpha-Synuclein locus triplication causes Parkinson's disease. *Science* **302**, 841.
- Sorrentino, Z.A., Giasson, B.I., and Chakrabarty, P. (2019). α -Synuclein and astrocytes: tracing the pathways from homeostasis to neurodegeneration in Lewy body disease. *Acta Neuropathol* **138**, 1–21.
- Shirasaka M., Kuwata K., Honda R. (2019). α -Synuclein chaperone suppresses nucleation and amyloidogenesis of prion protein. *Biochem Biophys Res Commun.* 2020 Jan 1;521(1):259-264. doi: 10.1016/j.bbrc.2019.10.120.
- Spatola, M., and Wider, C. (2014). Genetics of Parkinson's disease: the yield. *Parkinsonism Relat. Disord.* **20 Suppl 1**, S35-38.

- Spillantini, M.G., and Goedert, M. (2018). Neurodegeneration and the ordered assembly of α -synuclein. *Cell Tissue Res* 373, 137–148.
- Spillantini, M.G., Divane, A., and Goedert, M. (1995). Assignment of human alpha-synuclein (SNCA) and beta-synuclein (SNCB) genes to chromosomes 4q21 and 5q35. *Genomics* 27, 379–381.
- Spillantini, M.G., Schmidt, M.L., Lee, V.M., Trojanowski, J.Q., Jakes, R., and Goedert, M. (1997). Alpha-synuclein in Lewy bodies. *Nature* 388, 839–840.
- Spillantini, M.G., Crowther, R.A., Jakes, R., Hasegawa, M., and Goedert, M. (1998). alpha-Synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with lewy bodies. *Proc. Natl. Acad. Sci. U.S.A.* 95, 6469–6473.
- Stavitsky, K., Brickman, A.M., Scarneas, N., Torgan, R.L., Tang, M.-X., Albert, M., Brandt, J., Blacker, D., and Stern, Y. (2006). The progression of cognition, psychiatric symptoms, and functional abilities in dementia with Lewy bodies and Alzheimer disease. *Arch. Neurol.* 63, 1450–1456.
- Stravalaci, M., Bastone, A., Beeg, M., Cagnotto, A., Colombo, L., Di Fede, G., Tagliavini, F., Cantù, L., Del Favero, E., Mazzanti, M., *et al.* (2012). Specific Recognition of Biologically Active Amyloid- β Oligomers by a New Surface Plasmon Resonance-based Immunoassay and an *in Vivo* Assay in *Caenorhabditis elegans*. *J. Biol. Chem.* 287, 27796–27805.
- Stuchlik, A. (2014). Dynamic learning and memory, synaptic plasticity and neurogenesis: an update. *Front Behav Neurosci* 8, 106.
- Subramaniam, S.R., and Federoff, H.J. (2017). Targeting Microglial Activation States as a Therapeutic Avenue in Parkinson's Disease. *Front. Aging Neurosci.* 9, 176.
- Surendranathan, A., Rowe, J., and O'Brien, J. (2015). Neuroinflammation in Lewy Body Dementia. *Parkinsonism & Related Disorders* 21.
- Sutton, M.A., and Schuman, E.M. (2006). Dendritic Protein Synthesis, Synaptic Plasticity, and Memory. *Cell* 127, 49–58.
- Sveinbjornsdottir, S. (2016). The clinical symptoms of Parkinson's disease. *J. Neurochem.* 139 Suppl 1, 318–324.
- Walsh, J., Watson, N., and Kipnis, J. (2014). T cells in the central nervous system: Messengers of destruction or purveyors of protection? *Immunology* 141, 340–344.
- Takada, M., Tokuno, H., Nambu, A., and Inase, M. (1998). Corticostriatal input zones from the supplementary motor area overlap those from the contra- rather than ipsilateral primary motor cortex. *Brain Res* 791, 335–340.
- Tanaka, S., Ishii, A., Ohtaki, H., Shioda, S., Yoshida, T., and Numazawa, S. (2013). Activation of microglia induces symptoms of Parkinson's disease in wild-type, but not in IL-1 knockout mice. *J Neuroinflammation* 10, 907.
- Tang, Y., and Le, W. (2016). Differential Roles of M1 and M2 Microglia in Neurodegenerative Diseases. *Mol Neurobiol* 53, 1181–1194.

- Tang, S.-C., Arumugam, T.V., Xu, X., Cheng, A., Mughal, M.R., Jo, D.G., Lathia, J.D., Siler, D.A., Chigurupati, S., Ouyang, X., *et al.* (2007). Pivotal role for neuronal Toll-like receptors in ischemic brain injury and functional deficits. *Proceedings of the National Academy of Sciences* *104*, 13798–13803.
- Tanner, C.M., Kamel, F., Ross, G.W., Hoppin, J.A., Goldman, S.M., Korell, M., Marras, C., Bhudhikanok, G.S., Kasten, M., Chade, A.R., *et al.* (2011). Rotenone, Paraquat, and Parkinson's Disease. *Environ Health Perspect* *119*, 866–872.
- Theillet, F.-X., Binolfi, A., Bekei, B., Martorana, A., Rose, H.M., Stuiver, M., Verzini, S., Lorenz, D., van Rossum, M., Goldfarb, D., *et al.* (2016). Structural disorder of monomeric α -synuclein persists in mammalian cells. *Nature* *530*, 45–50.
- Tiraboschi, P., Salmon, D.P., Hansen, L.A., Hofstetter, R.C., Thal, L.J., and Corey-Bloom, J. (2006). What best differentiates Lewy body from Alzheimer's disease in early-stage dementia? *Brain* *129*, 729–735.
- Togo, T., Iseki, E., Marui, W., Akiyama, H., Uéda, K., and Kosaka, K. (2001). Glial involvement in the degeneration process of Lewy body-bearing neurons and the degradation process of Lewy bodies in brains of dementia with Lewy bodies. *Journal of the Neurological Sciences* *184*, 71–75.
- Tolosa, E., and Compta, Y. (2006). Dystonia in Parkinson's disease. *J. Neurol.* *253 Suppl 7*, VII7-13.
- Tolosa, E., Wenning, G., and Poewe, W. (2006). The diagnosis of Parkinson's disease. *Lancet Neurol* *5*, 75–86.
- Tompkins, M.M., and Hill, W.D. (1997). Contribution of somal Lewy bodies to neuronal death. *Brain Res.* *775*, 24–29.
- Tong, J., Ang, L.-C., Williams, B., Furukawa, Y., Fitzmaurice, P., Guttman, M., Boileau, I., Hornykiewicz, O., and Kish, S.J. (2015). Low levels of astroglial markers in Parkinson's disease: relationship to α -synuclein accumulation. *Neurobiology of Disease* *82*, 243–253.
- Tremblay, M.-E., Stevens, B., Sierra, A., Wake, H., Bessis, A., and Nimmerjahn, A. (2011). The Role of Microglia in the Healthy Brain. *Journal of Neuroscience* *31*, 16064–16069.
- Trudler, D., Farfara, D., and Frenkel, D. (2010). Toll-Like Receptors Expression and Signaling in Glia Cells in Neuro-Amyloidogenic Diseases: Towards Future Therapeutic Application. *Mediators of Inflammation* *2010*, 1–12.
- Tsigelny, I.F., Sharikov, Y., Wrasidlo, W., Gonzalez, T., Desplats, P.A., Crews, L., Spencer, B., and Masliah, E. (2012). Role of α -synuclein penetration into the membrane in the mechanisms of oligomer pore formation. *FEBS J.* *279*, 1000–1013.
- Tysnes, O.-B., and Storstein, A. (2017). Epidemiology of Parkinson's disease. *J Neural Transm (Vienna)* *124*, 901–905.
- Uéda, K., Fukushima, H., Masliah, E., Xia, Y., Iwai, A., Yoshimoto, M., Otero, D.A., Kondo, J., Ihara, Y., and Saitoh, T. (1993). Molecular cloning of cDNA encoding an unrecognised component of amyloid in Alzheimer disease. *Proc. Natl. Acad. Sci. U.S.A.* *90*, 11282–11286.

- Valente, E.M., Bentivoglio, A.R., Dixon, P.H., Ferraris, A., Ialongo, T., Frontali, M., Albanese, A., and Wood, N.W. (2001). Localisation of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. *Am. J. Hum. Genet.* *68*, 895–900.
- Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M.K., Harvey, K., Gispert, S., Ali, Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., *et al.* (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* *304*, 1158–1160.
- Villar-Piqué, A., Lopes da Fonseca, T., and Outeiro, T.F. (2016). Structure, function and toxicity of alpha-synuclein: the Bermuda triangle in synucleinopathies. *J. Neurochem.* *139 Suppl 1*, 240–255.
- Virmani, T., Moskowitz, C.B., Vonsattel, J.-P., and Fahn, S. (2015). Clinicopathological characteristics of freezing of gait in autopsy-confirmed Parkinson's disease. *Mov. Disord.* *30*, 1874–1884.
- Wakabayashi, K., Tanji, K., Odagiri, S., Miki, Y., Mori, F., and Takahashi, H. (2013). The Lewy body in Parkinson's disease and related neurodegenerative disorders. *Mol. Neurobiol.* *47*, 495–508.
- Wales, P., Pinho, R., Lázaro, D.F., and Outeiro, T.F. (2013). Limelight on alpha-synuclein: pathological and mechanistic implications in neurodegeneration. *J Parkinsons Dis* *3*, 415–459.
- Wang, J., Chen, Z., Walston, J.D., Gao, P., Gao, M., and Leng, S.X. (2019). Interferon- γ Potentiates α -Synuclein-induced Neurotoxicity Linked to Toll-like Receptors 2 and 3 and Tumor Necrosis Factor- α in Murine Astrocytes. *Mol Neurobiol.*
- Wijeyekoon, R.S., Kronenberg-Versteeg, D., Scott, K.M., Hayat, S., Jones, J.L., Clatworthy, M.R., Floto, R.A., Barker, R.A., and Williams-Gray, C.H. (2018). Monocyte Function in Parkinson's Disease and the Impact of Autologous Serum on Phagocytosis. *Front. Neurol.* *9*, 870.
- Winner, B., Jappelli, R., Maji, S.K., Desplats, P.A., Boyer, L., Aigner, S., Hetzer, C., Loher, T., Vilar, M., Campioni, S., *et al.* (2011). In vivo demonstration that alpha-synuclein oligomers are toxic. *Proc. Natl. Acad. Sci. U.S.A.* *108*, 4194–4199.
- Wu, D.C., Jackson-Lewis, V., Vila, M., Tieu, K., Teismann, P., Vadseth, C., Choi, D.-K., Ischiropoulos, H., and Przedborski, S. (2002). Blockade of Microglial Activation Is Neuroprotective in the 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine Mouse Model of Parkinson Disease. *J. Neurosci.* *22*, 1763–1771.
- Xilouri, M., Brekk, O.R., and Stefanis, L. (2013). α -Synuclein and protein degradation systems: a reciprocal relationship. *Mol. Neurobiol.* *47*, 537–551.
- Xu, J., Zhong, N., Wang, H., Elias, J.E., Kim, C.Y., Woldman, I., Pifl, C., Gygi, S.P., Geula, C., and Yankner, B.A. (2005). The Parkinson's disease-associated DJ-1 protein is a transcriptional co-activator that protects against neuronal apoptosis. *Hum. Mol. Genet.* *14*, 1231–1241.
- Xu, Y.-J., Mei, Y., Qu, Z.-L., Zhang, S.-J., Zhao, W., Fang, J.-S., Wu, J., Yang, C., Liu, S.-J., Fang, Y.-Q., *et al.* (2018). Ligustilide Ameliorates Memory Deficiency in APP/PS1

Transgenic Mice via Restoring Mitochondrial Dysfunction. *BioMed Research International* 2018, 1–15.

Yu, S., Li, X., Liu, G., Han, J., Zhang, C., Li, Y., Xu, S., Liu, C., Gao, Y., Yang, H., *et al.* (2007). Extensive nuclear localisation of alpha-synuclein in normal rat brain neurons revealed by a novel monoclonal antibody. *Neuroscience* 145, 539–555.

Yu, S., Zuo, L., Wang, F., Chen, Z., Hu, Y., Wang, Y., Wang, X., and Zhang, W. (2014). Potential biomarkers relating pathological proteins, neuroinflammatory factors and free radicals in PD patients with cognitive impairment: a cross-sectional study. *BMC Neurol* 14, 113.

Zarranz, J.J., Alegre, J., Gómez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atarés, B., *et al.* (2004). The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann. Neurol.* 55, 164–173.

Zhang, G., Xia, Y., Wan, F., Ma, K., Guo, X., Kou, L., Yin, S., Han, C., Liu, L., Huang, J., *et al.* (2018). New Perspectives on Roles of Alpha-Synuclein in Parkinson's Disease. *Front Aging Neurosci* 10, 370.

Zhang, L., Shimoji, M., Thomas, B., Moore, D.J., Yu, S.-W., Marupudi, N.I., Torp, R., Torgner, I.A., Ottersen, O.P., Dawson, T.M., *et al.* (2005). Mitochondrial localisation of the Parkinson's disease related protein DJ-1: implications for pathogenesis. *Hum. Mol. Genet.* 14, 2063–2073.

Zhang, Z.-X., Dong, Z.-H., and Román, G.C. (2006a). Early descriptions of Parkinson disease in ancient China. *Arch. Neurol.* 63, 782–784.

Zhang, L., Dawson, V.L., and Dawson, T.M. (2006b). Role of nitric oxide in Parkinson's disease. *Pharmacology & Therapeutics* 109, 33–41.

Zhou, W., Milder, J.B., and Freed, C.R. (2008). Transgenic mice overexpressing tyrosine-to-cysteine mutant human alpha-synuclein: a progressive neurodegenerative model of diffuse Lewy body disease. *J. Biol. Chem.* 283, 9863–9870.